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(54) Title: PLANT–DERIVED RESISTANCE GENE

(57) Abstract

Disclosed are nucleic acids encoding polypeptides which are capable of conferring extreme resistance (ER) against, and being triggered by, plant pathogens such as viruses (e.g. PVX and related isolates). Preferred nucleic acids encode the Rx polynucleotide from *Solanum tuberosum*, or a variety of homologues (naturally occurring or derivatives) thereof, such as 111h1; 221h2; Ac15; Ac64; K39.hom. Particular methods of activating resistance by using combinations of resistance gene and elicitor are also disclosed, which in certain cases lead to a hypersensitive response. Further aspects of the invention include specific primers, vectors, host cells, polypeptides, antibodies and transgenic plants, plus methods of producing and employing these, in particular for influencing a resistance trait in a plant.

MAYAAVTSLMRTIHQSMELTGCDLQPFYEKLKSLRAI
LEKSCNIMGDHEGLTILEVEIVEVAYTTEDMVDSESR
NVFLAQNLEERSRAMWEIFFVLEQALECIDSTVKQWM
ATSDSMKDLKPQTSSLVSLPEHDVEQPENIMVGRENE

FEMMLDQLARGGRELEVVSIVGMGGIGKTTLAT — kinase motiff 1a

KLYSDPCIMSRFDIRAKATVSQEYCVRNVLLGLLSLT
SDEPDDQLADRLQKHLKGRRYLVVIDDIWTTEAW — kinase motiff 2

DDIKLCFPDCYNGSRILLTTRNVEVAEYASSGKP — kinase motiff 3a

PHHMRLMNFDESWNLLHKKIFEKEGSYSPEFENIGKQ
IALKCGGLPLAITVIAGLLSKMGQRLDEWQRIG — R gene signature 1

ENVSSVVSTDPEAQCMRVLALSYHHLPSH
LKPCFLYFAIFTEDEQISVNELVELWPVEGFLNE — R gene signature 2

EEGKSIEEVATTCINELIDRSLIFIHNFSFRGTIESCG
MHDVTRELCLREARN — R gene signature 3

MNFVNVIRG KSDQNSCAQS MQRSFKSRSR
IR IHKVEELAWCRNSEAHS
IIMLGGFECVIL
ELSFKLVRVLDLGLN TW PIFPSG
VLSLIHLRYLSLRFNPCLQQYQGSKEAVPSSIIDIPLS
ISSLCYLQTFKLNL PFPSYYPFILPSE
ILTMPQLRTLCMGWN YLRSHEPTENRLV
LKNLQCLNQLNPRYCTGSF
FRLFPNLKKLQVFGVPEDFRNSQDLYD
FRYLYQLEELTFRLYYPYAACFLKNTAPSGSTQDPLRF
QTELLH KEIDEGGTAPPTLLLPPP
DAFPQNLKSLIFRGEFSVAWKDLSI
VGKLPKLEVLILSWNAFIGKEWEVV
EEGFPHLKFLFLDD VYIRYWRAS
SDHFPVLERVILRDCRNLD SIPRD
FADITLALIDIDYC

{axx axx L xx Lx Lxx N xxa xxx aPxx} LRR consensus
C
T

QQSVVNSAKQIQQDIQDNYGSSIEV — Amide(QN)-rich

HTRHLFIPK — Basic (+)

SVTTVEDDDDSVTTDEDDDDDDFEKEVASCRNNVE — Acidic (-)

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PLANT-DERIVED RESISTANCE GENE

TECHNICAL FIELD

The present invention relates to the *Rx* resistance gene from potato. It further relates to methods and materials employing the gene, and processes for identifying or producing other related genes. It also relates generally to methods for identifying novel genes or markers within clusters of repetitive DNA sequences.

PRIOR ART

Rx is a resistance gene from potato conferring extreme resistance against potato virus X (PVX). *Rx* genes in potato are widely used in breeding programmes to confer resistance against PVX because:

1) *Rx* is highly durable with only one natural isolate able to overcome the resistance.

2) The resistance is extreme resistance according to the classification of Cooper (Cooper and Jones, 1983).

Extreme resistance (ER) means that there is no visible indication of a resistance response on *Rx* plants inoculated with PVX and accumulation of PVX is suppressed, even in the inoculated cells.

In contrast, other types of disease resistance (including the virus resistance conferred by the previously cloned N gene in tobacco) are associated with a hypersensitive

response (HR) which causes necrosis at the site of inoculation. Activation of these other types of resistance mechanisms is normally delayed so that the virus accumulates in the inoculated cell at the same rate in cells of the resistant and the susceptible plants (see WO 95/31564 (Gatsby Charitable Foundation) for a general discussion of HR).

A further difference between *Rx*-mediated resistance and HR type resistance is that the *Rx*-mediated resistance is active in protoplasts wherein it suppresses viral replication or promotes degradation of the viral RNA. In contrast HR resistance is not expressed in protoplasts but is thought to be a tissue-related phenomenon requiring cell-to-cell contact (Adams et al, 1986).

The *Rx*-mediated resistance against PVX is thought to conform to an elicitor-receptor model. According to the model there are two phases in the Rx resistance mechanism: a recognition phase that is believed to be highly specific for potato virus X and a response phase that prevents accumulation of a broad spectrum of plant viruses, including those taxonomically unrelated to PVX (Köhm et al., 1993). Published work has also demonstrated that the PVX coat protein (CP) is the elicitor of Rx-mediated resistance (Bendahmane et al., 1995; Goulden et al., 1993).

Although the activity of the Rx phenotype has been studied, little is known about the gene responsible for it.

Ritter et al (1991) disclosed that there are two *Rx* loci, *Rx1* and *Rx2* (on chromosomes XII and V respectively). These

loci function independently of each other and have the same specificity for the PVX CP. It has been suggested that *Rx* may have been introgressed into potato cultivars from a wild *Solanum* accession (see also Arntzen et al (1994)).

Bendahmane et al (1997) disclosed flanking AFLP markers on Chromosome XII (IPM3 and IPM4) in the vicinity of *Rx* which span an interval of 0.23 cM. However no accurate estimate was made of the physical distance that this represents, and hence of whether or not a positional cloning approach based on these markers was plausible.

Indeed such a map based approach may be expected to be complicated by the tetraploid nature of the potato genome and the chromosomal duplication referred to above. Additionally, the fact that *Rx* lies within an introgressed chromosomal segment would also be expected to hinder map based cloning efforts because recombination is believed to be suppressed in such regions (see Liharska et al (1996)).

Notably, there are no published reports of genes which have been cloned from potato using map-based approaches despite the high resolution mapping of several loci in the potato genome (Ballvora *et al.*, 1995; Brigneti *et al.*, 1997; De Jong *et al.*, 1997; Meksem *et al.*, 1995). In fact, although there have been some isolated examples of map-based cloning from crop plants (Buschges et al., 1997; Dixon et al., 1996; Martin, 1993) most plant applications of this technique have been in the model plant *Arabidopsis thaliana*.

DISCLOSURE OF THE INVENTION

The present inventors have applied a number of innovative strategies to identify a bacterial artificial chromosome (BAC) clone that spans *Rx*. This approach was successful despite the suppression of recombination and depletion of AFLP and RFLP markers in the vicinity of *Rx*, and also various other complications arising from repetitive sequences in the *Rx* region and the highly polymorphic nature of the potato genome.

Briefly, in order to clone the *Rx* locus in potato they prepared a BAC library from a tetraploid plant carrying *Rx* in the duplex condition (*Rx, Rx, rx,rx*). BAC clones isolated from either side of *Rx* including some in which there was a high frequency of recombination (approximately 180 kb cM^{-1}). However, the closest markers to *Rx* in the cloned DNA were separated by single recombination events on either side of *Rx*. To bridge the gap they exploited the finding that the BAC clones on the right side of *Rx* appeared to contain resistance gene homologues. Anticipating that there may be duplicated resistance gene homologues in the vicinity of *Rx* they used low stringency PCR conditions to identify additional markers.

One of these markers was completely linked to *Rx* in the mapping population and was used to isolate a further BAC (BAC77) which could not be identified with known, existing, *Rx*-flanking markers.

Two criteria suggested that BAC77 spanned *Rx*. There was a chromosomal recombination in one plant of the mapping population that separated the BAC77 right end from *Rx*. On the other side of *Rx* it was found that the BAC77 left end

was outside the region of DNA carrying *Rx* that had been introgressed into potato cv Amaryl from *Solanum tuberosum* ssp. *andigena* CPC1673. The fact that BAC77 did indeed span *Rx* was shown using novel, transient expression assays, and by transgenic expression in *Nicotiana tabacum*, *Nicotiana benthamiana*, *Lycopersicon esculentum* and potato.

This is the first example of a gene capable of conferring ER being isolated. The *Rx* gene has been sequenced and the expression product shown to share some motifs with other, known, resistance proteins, as well as having some distinctive sequence regions. The inventors have demonstrated that it can be used to introduce PVX resistance into plants, including *L. esculentum*, *N. tabacum*, *N. benthamiana* and potato.

Interestingly, it has been demonstrated that constitutive co-expression of the *Rx* gene and the PVX elicitor can actually cause an HR. A demonstration of this came from constitutive expression of the PVX CP elicitor, under the control of a 35S promoter, in Rx plants. Such constitutive expression leads to cell death - production of elicitor can not be arrested by the action of Rx mediated resistance before it reaches a critical level. In contrast, when the coat protein is produced as a product of the PVX genome the early activation of the Rx-mediated resistance is thought to suppress the virus so that the coat protein would not attain the critical level required for elicitation of the HR. Other results herein show that the Rx ER response is epistatic to the HR response, whereby the traits can be manipulated independently. The HR observations have also been exploited in an innovative assay for PVX elicitors or

Rx variants.

Thus portions of the *Rx* sequence can be used to identify *Rx* homologues, some of which may be closely-linked to the *Rx* locus.

Further, the *Rx* gene (or modifications or homologues thereof) can be used to engineer resistance traits, preferably broad spectrum ER, into plants using a variety of innovative formats.

It has also been discovered by the present inventors that *Rx* can be activated by certain non-PVX viruses, particularly those comprising coat proteins sharing sequence homology with that of PVX. Examples within this group include (but are not limited to) Narcissus mosaic virus (NMV); Nandina virus X (NVX); Viola mosaic virus (VMV); Cymbidium mosaic virus (CyMV); Poplar mosaic virus (PopMV) and White clover mosaic virus (WClMV). *Rx* can be used to offer specific protection against this group.

These and other aspects of the present invention will now be discussed in more detail.

According to a first aspect of the present invention there is provided a nucleic acid molecule encoding a polypeptide which is capable of conferring extreme resistance against a pathogen, such as a plant virus, in a plant into which said polypeptide is expressed.

As discussed above, extreme resistance, implies the absence of a visible HR in the presence of the host pathogen.

Nucleic acid molecules according to the present invention may be provided in recombinant form or free or substantially free of nucleic acid or genes of the species of interest or origin other than the sequence encoding a polypeptide with the required function. The nucleic acid molecules (and their encoded polypeptide products) may also be (i) isolated and/or purified from their natural environment (although not necessarily in pure form *per se*), or (ii) in substantially pure or homogeneous form.

Nucleic acid according to the present invention may include cDNA, RNA, genomic DNA and may be wholly or partially synthetic ('constructs'). Where a DNA sequence is specified, e.g. with reference to a figure, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed.

Also encompassed is the complement of the various disclosed sequences, which may be used in probing experiments, or in down-regulation of the sequence.

A particular aspect of the invention is nucleic acid having the sequence all or part of the sequence shown in Annex 1 (Seq ID No 1) including (where appropriate) both coding and/or non-coding regions.

This sequence was taken from the BAC subclone which conferred Rx mediated resistance in the Examples below.

Within Seq ID No 1 there is apparently a large open reading frame (ORF). Subsequent comparison of the genomic DNA sequence with the sequence of cDNAs revealed that the gene

contains three exons and two introns in the 3' end (234 bp and 111 bp). The introns are marked as shown in Annex 1. The final exon contains only two nucleotides of coding sequence plus the TAG stop codon and the 3' non-coding region of mRNA.

The putative Rx polypeptide sequence is shown in Fig 1 - designated Seq ID No 2). Rx appears to contain 937 residues and have a molecular weight of 107.5 kD.

Particular nucleic acids of this aspect of the invention include those encoding the Rx protein product and cDNA, believed to be base 2249-5404 excluding the introns marked as shown (4945-5178 and 5290-5400 inclusive). The Rx-coding (cDNA) nucleic acid sequence is designated Seq ID No 3. This is shown as Annex II.

Surprisingly the primary structure of Rx is similar to that of the NBS-LRR (Jones and Jones, 1997) class of R proteins, in which the resistance is associated with an HR. The highest degree of similarity is between Rx and a subclass of NBS-LRR resistance proteins represented by Rps2, Rpm1 and Prf (Jones and Jones, 1997). These Arabidopsis and tomato proteins contain a putative four to six heptad amphipathic leucine zipper (LZ) motif at the N-terminus (Jones and Jones, 1997). A putative leucine zipper is also present in the N-terminal region of Rx. However this does not fit the leucine zipper consensus was well as the corresponding motif in Rps2 (Mindrinos et al, 1994).

Comparisons are shown in Fig 2. As in the other *R* gene products, the putative NBS domain (domain 11; Figure 1) of

Rx comprises three motifs: kinase 1A or 'P-loop' (residues 168-180), kinase 2 (residues 237-247), and kinase 3a (residues 265-273). In Rx, the putative NBS is followed by a domain with unknown function that includes GLPL, CFLY and the MHD motifs. These motifs are characteristic of all NBS-LRR *R* gene products thus far identified (Hammond-Kosack and Jones, 1997; van der Biezen and Jones, 1998). The putative LRR domain of Rx (residues 473-868) comprises 14-16 imperfect copies of the LRR motif. This motif shows a good match to the cytoplasmic LRR consensus sequence motif (Jones and Jones, 1997) and most closely resembles the LRR domain of the tomato Prf protein (Salmeron et al., 1996).

The C-terminal part of Rx contains three unique motifs with unknown functions: an amide-rich region (residues 869-893), a short basic region (residues 894-902) and an 'acidic tail' region (residues 903-937). The acidic tail is encoded entirely within the short second exon of Rx. This domain is rich in aspartic and glutamic acid residues and contains two copies of 10 amino acid residues in direct repeat. These features are not present in any previously described products of *R* genes.

Generally speaking, the sequence conservation between the Rx and other disease resistance genes cloned in other systems was found to be very low and was mostly in the NBS domain in the N terminal part of Rx. Referring to Fig 2, the sequence identity between Rx and PRF, RPS2, RPM1 and I2C-1 was found to be 23.1, 15.2 ,15.3 and 17.8% respectively. In contrast, within the Rx class of disease resistance genes of the present invention (see Examples below) the conservation was very high. For instance the sequence identity shared by Rx and 111h1, 222h2, Ac15, Ac64 and k39.hom is 93, 84.9,

97.3, 97.3 and 92.1% respectively. This demonstrated similarity within the group allows the cloning of yet further Rx-type resistance genes using the sequences disclosed herein, either directly, or to design degenerate primers.

Thus in a further aspect of the invention there are disclosed active, homologous, variants of the *Rx* sequences provided, which may for instance be mutants or other derivatives, or naturally occurring Rx homologues such as allelic variants, paralogues (from the same species, but at a different location e.g. pseudoalleles at linked loci), or orthologues (related genes from different species). Examples of these are shown below.

In each case the variant encodes a product which is homologous (similar) to Rx, which may be isolated or produced on the basis of that sequence, and is capable of conferring pathogen resistance against one or more pathogens.

Resistance gene activity can be tested by conventional methods known in the art, as appropriate to the nature of the resistance being investigated. Example methods can be found in the following publications: bacterial (Grant et al, 1995); fungal (Dixon et al, 1996; Jones, 1994; Thomas et al, 1997); nematode and viral (Whitham et al, 1994).

Typically, activity is tested by complementation of trait in a plant. This can be achieved by coupling the putative active variant to a promoter and terminator for expression in plants and transforming it into a 'susceptible' plant

that lacks a given resistance trait. The activity of the Rx variant is then confirmed by challenge with the appropriate pathogen.

Alternatively a transient expression assay can be used to test for activation of the Rx variant analogous to the assay described in the Examples below or that used by Mindrinos et al (1994). Briefly, the putative active Rx variant is co-expressed from a plasmid with a pathogen-derived gene which is an elicitor of the resistance specified by the putative Rx homologue (in the case of Rx this can be PVX-CP) and a reporter gene (e.g. GUS or CP). If the variant is activated by the continuous expression of the pathogen derived gene, then an HR would result and the reporter gene activity would be abolished. If no activity was initiated, then the reporter gene would be detectable.

Similarity or homology between the variant and Rx may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art, or, and this may be preferred, the standard program BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). Comparisons herein have used DNASTAR software using the CLUSTAL method with PAM250 residue weight table (gap penalty 10, gap length 10).

Homology (or similarity, or identity) may be at the nucleotide sequence and/or the expressed amino acid sequence level. Preferably, the nucleic acid and/or amino acid sequence shares homology with the coding sequence or the

sequence encoded by the nucleotide sequence of Annex I or other sequences set out herein, preferably at least about 50%, or 60%, or 70%, or 80% homology, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% homology.

Homology may be over the full-length of the relevant sequence shown herein, or may more preferably be over a contiguous sequence of about or greater than about e.g. 20, 100, 200, 300, 500, 600 or more amino acids or codons, compared with the relevant amino acid sequence or nucleotide sequence as the case may be.

There are believed to be more than 20 homologues of *Rx* in the potato genome. It is likely that one or more of these homologues are R genes against viruses, fungi, bacteria or nematodes.

Naturally occurring Rx variants may be isolated, in the light of the present disclosure, without burden from any suitable source (e.g. genomic or cDNA). The putative resistance genes can be obtained using materials (e.g. primers or probes) based on regions peculiar to *Rx*, for instance designated 'Rx gene signature' in Fig 1. It should be noted that previously identified sequence motifs that characterise resistance genes (see e.g. Kanazin et al., 1996; Leister et al., 1996; Leister et al., 1998) are not useful for identification of these *Rx* variants because they do not discriminate between the *Rx* variants and homologues of other resistance gene homologues in the genome.

Thus a further aspect of the present invention provides a method of identifying and/or cloning homologous *Rx* variants

from a plant, which method employs all or part of a nucleotide sequences as described above.

Thus in one embodiment, nucleotide sequence information provided herein may be used in a data-base (e.g. of ESTs, or STSs, or other genomic sequence information) search to find homologous sequences, expression products of which can be tested for pathogen resistance activity e.g. using methods based on the transient assays of the present invention, or conventional phenotype assays in transgenic plants.

Alternatively, probes based on the sequence may be used e.g. in southern blotting. For instance DNA may be extracted from cells taken from plants displaying the appropriate resistance trait and digested with different restriction enzymes. Restriction fragments may then be separated (e.g. by electrophoresis on an agarose gel) before denaturation and transfer to a nitrocellulose filter. Labelled probe may be hybridised to the DNA fragments on the filter and binding determined.

Preliminary experiments may be performed by hybridising under low stringency conditions. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further.

For example, hybridizations may be performed, according to the method of Sambrook et al. (below) using a hybridization solution comprising: 5X SSC (wherein 'SSC' = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7), 5X Denhardt's reagent, 0.5-1.0% SDS, 100 μg/ml denatured, fragmented

salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes - 1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989):

T_m = 81.5°C + 16.6Log [Na+] + 0.41 (% G+C) - 0.63 (% formamide) - 600/#bp in duplex

As an illustration of the above formula, using [Na+] = [0.368] and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain. Other suitable conditions include, e.g. for detection of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in

0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include amplification using PCR (including, where appropriate, RACE PCR), RN'ase protection and allele specific oligonucleotide probing.

The identification of successful hybridisation is followed by isolation of the nucleic acid which has hybridised, which may involve one or more steps of PCR or amplification by cloning in a vector that replicates in a suitable host.

In each case, if need be clones (e.g. lambda, cosmid, plasmid, BACs, biBACS) or fragments identified in the search can be extended or supplemented. For instance if it is suspected that they are incomplete, the original DNA source (e.g. a clone library, mRNA preparation etc.) can be revisited to isolate missing portions e.g. using sequences, probes or primers based on that portion which has already been obtained to identify other clones containing overlapping sequence (see e.g. "Principles of Genome Analysis" by S B Primrose (1995) Pub. Blackwell Science Ltd, Oxford, UK).

The genes are then tested for functionality as described above. Thus one scheme for isolating *Rx* homologues is as follows:

I) produce a population in which a resistance trait is segregating.

II) PCR amplify DNA from individual members of the population with primers based on the sequence of *Rx* (but not from the R gene conserved motifs).

III) test the PCR products (either by direct sequence analysis or restriction enzyme digestion) for sequence polymorphism that cosegregates with the R trait. Identify an appropriate polymorphic marker sequence.

IV) Isolate the complete coding sequence of the polymorphic gene. This could be done from an appropriate cloned library or by amplifying it using primers from the 5' and 3' extremes of Rx. In each case the identified polymorphic PCR product, or sequence information provided by it, is used to identify the gene.

Resistance gene coding activity is then tested as described above. A variation on this procedure would employ bulked segregants in an intermediate stage, as described previously (Michelmore et al., 1991).

A more specific approach is based on the understanding that homologous *Rx*-genes may be linked in clusters. Clustering of *R*-genes in potato has already been reported (Leister et al. 1996; De Jong et al. 1997). One of the large *R*-gene clusters

is on the short arm of potato chromosome *5*. This cluster comprises of at least four *R* loci: *R1* conferring resistance to *Phytophthora infestans*, *Nb* conferring HR type resistance to potato virus X (PVX) and *Gpa* and *Grp1* conferring resistance to the potato cyst nematode.

It is now clear that a number of the genes and DNA sequences linked to *Rx* represent *Rx*-homologues. It is likely that one or more of these homologues are R genes against viruses, fungi, bacteria or nematodes. For instance, *Gpa2* (which bestows a specific resistance response to a small set of populations of the potato cyst nematode *Globodera pallida*) is believed to be tightly linked to the *Rx* locus on chromosome *12*.

As set out in the Examples below, a functional homologue has been isolated from *Solanum acuale*, using primers based on the sequence of the cloned Rx.

The *S. acaule Rx*-homologue is likely to be on chromosome V linked to *Rx2* and, using the agrobacterium infiltration assay, has been shown to function as a resistance gene against PVX. This result shows that the sequence of Rx can be used to isolate other resistance genes without reference to the conserved motifs that have been used previously to define resistance gene homologues (Leister et al., 1996).

Other, linked, *Rx* variants (providing different R traits) may be isolated essentially as set out above, but wherein the DNA used for the initial amplification step is taken from members of the population in which the required R trait co-segregates with *Rx* itself (or a previously identified Rx

variant).

It has been noted by the present inventors that the sequence of Rx is similar to the sequence of the otherwise unrelated Rps2 that confers resistance in Arabidopsis against a bacterial pathogen. In the light of this information it appears that the sequence of Rx could be modified e.g. by site-directed or random mutation, to produce Rx mutants or other derivatives which can confer resistance against (i.e. is switched on by) pathogens that are quite different from PVX. This can be achieved as described below, with Rx mutants being tested with the transient expression assay methods described above.

Preferably the nucleic acid molecule which is the mutant or other derivative is generated either directly or indirectly (e.g. via one or amplification or replication steps) from an original nucleic acid corresponding to all or part of the sequence shown in Seq ID No 1 or other sequences disclosed herein.

Thus a further aspect of the present invention is a method of producing a nucleic acid encoding an Rx derivative comprising the step of modifying a nucleic acid encoding Rx.

The derivative may include changes to the nucleic acid which make no difference to the encoded amino acid sequence (i.e. 'degeneratively equivalent').

Changes to a sequence, to produce a mutant or derivative, may be by one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid,

leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded polypeptide.

In addition to one or more changes within the Rx sequence, a variant nucleic acid may encode an amino acid sequence including additional amino acids at the C-terminus and/or N-terminus.

Specifically included are parts or fragments (however produced) corresponding to portions of the sequences provided, and which encode polypeptides having biological activity - for instance pathogen resistance or the ability to raise Rx-binding antibodies.

Generally speaking, changes may be desirable for a number of reasons, including introducing or removing the following features: restriction endonuclease sequences; codon usage; other sites which are required for post translation modification; cleavage sites in the encoded polypeptide; motifs in the encoded polypeptide for glycosylation, lipoylation etc. Leader or other targeting sequences may be added to the expressed protein to determine its location following expression. All of these may assist in efficiently cloning and expressing an active polypeptide in recombinant form (as described below).

Other desirable mutation may be random or site directed mutagenesis in order to alter the activity (e.g. specificity) or stability of the encoded polypeptide.

As is well-understood, homology at the amino acid level is determined in terms of amino acid similarity or identity.

Similarity allows for conservative variation, i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. As is well known to those skilled in the art, altering the primary structure of a polypeptide by a conservative substitution may not significantly alter the activity of that peptide because the side-chain of the amino acid which is inserted into the sequence may be able to form similar bonds and contacts as the side chain of the amino acid which has been substituted out. This is so even when the substitution is in a region which is critical in determining the peptides conformation.

Also included are homologues having non-conservative substitutions. As is well known to those skilled in the art, substitutions to regions of a peptide which are not critical in determining its conformation may not greatly affect its activity because they do not greatly alter the peptide's three dimensional structure. In regions which are critical in determining the peptides conformation or activity such changes may alter the properties of the polypeptide. Indeed, changes such as those described above may confer slightly advantageous properties on the peptide e.g. altered stability or specificity, in particular broader specificity. Mutants having these properties can then be selected as described above.

Other methods may include mixing or incorporating sequences from related resistance genes into the Rx sequence. For example restriction enzyme fragments of Rx could be ligated

together with fragments of an Rx homologue or even of an unrelated gene to generate recombinant versions of Rx. An alternative strategy for modifying Rx would employ PCR as described above (Ho et al., 1989) or DNA shuffling (Crameri et al., 1998).

Thus the methods of the invention, described above, may include hybridisation of one or more (e.g. two) probes or primers based on the *Rx* sequence either to screen for Rx homologues or to produce *Rx* derivatives. Such probes or primers form a further part of the present invention.

An oligonucleotide for use in probing or PCR may be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 nucleotides in length. For optimum specificity and cost effectiveness, primers of 16-24 nucleotides in length may be preferred. Those skilled in the art are well versed in the design of primers for use processes such as PCR. If required, probing can be done with entire restriction fragments of the gene disclosed herein which may be 100's or even 1000's of nucleotides in length.

In one aspect of the present invention, the nucleic acid described above is in the form of a recombinant and preferably replicable vector.

"Vector" is defined to include, inter alia, any plasmid, cosmid, phage or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform prokaryotic or eukaryotic host either by

integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

Specifically included are shuttle vectors by which is meant a DNA vehicle capable, naturally or by design, of replication in two different host organisms, which may be selected from actinomycetes and related species, bacteria and eucaryotic (e.g. higher plant, mammalian, yeast or fungal cells).

A vector including nucleic acid according to the present invention need not include a promoter or other regulatory sequence, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate promoter or other regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial, or plant cell. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic DNA, this may contain its own promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA).

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

Thus this aspect of the invention provides a gene construct, preferably a replicable vector, comprising a promoter operatively linked to a nucleotide sequence provided by the present invention, such as the *Rx* gene, or a variant (e.g mutant, derivative or allele) thereof.

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al*, 1989, Cold Spring Harbor Laboratory Press.

Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis (see above), sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

In one embodiment of this aspect of the present invention provides a gene construct, preferably a replicable vector, comprising an inducible promoter operatively linked to a nucleotide sequence provided by the present invention.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. The preferable situation is where the level of expression increases upon application of the relevant stimulus by an amount effective to alter a phenotypic characteristic. Thus an inducible (or "switchable") promoter may be used which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or switched on) to a level which brings about the desired phenotype.

Particularly of interest in the present context are plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Bevan (Nucl. Acids Res. 12, 8711-8721 (1984)) and Guerineau and

Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148).

Since the natural *in vivo* activation of Rx is believed to be post-transcriptional, it may be preferred that a constitutive promoter is used.

Suitable promoters which operate in plants include the Cauliflower Mosaic Virus 35S (CaMV 35S) gene promoter that is expressed at a high level in virtually all plant tissues (Benfey et al, 1990a and 1990b); the cauliflower meri 5 promoter that is expressed in the vegetative apical meristem as well as several well localised positions in the plant body, e.g. inner phloem, flower primordia, branching points in root and shoot (Medford, 1992; Medford et al, 1991) and the *Arabidopsis thaliana* LEAFY promoter that is expressed very early in flower development (Weigel et al, 1992). Other promoters include the rice actin promoter.

Alternatively an inducible promoter may be used. For instance the GST-II-27 gene promoter, which has been shown to be induced by certain chemical compounds which can be applied to growing plants. The promoter is functional in both monocotyledons and dicotyledons. It can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, cotton; cereals such as wheat, barley, rice, maize, sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, and melons; and vegetables such as carrot, lettuce, cabbage and onion. The GST-II-27 promoter is also suitable for use in a

variety of tissues, including roots, leaves, stems and reproductive tissues. Other promoters include the patatin promoter (tubers), ubiquitin promoter (wheat embryos).

The promoter may include one or more sequence motifs or elements conferring developmental and/or tissue-specific regulatory control of expression.

Thus the vectors of the present invention may include the Rx gene or a variant thereof, in addition to various sequences required to give them replicative, integrative and/or expression functionality. Such vectors can be used, for instance, to make plants into which they are introduced resistant to PVX or other viruses.

If it is desired to induce broader-spectrum resistance, various further options are available in the light of the present disclosure:

(a) Modify the *Rx* sequence, to produce mutants or other derivatives as discussed above, such that its effect can be initiated by elicitors or pathogens other than PVX alone or the other natural elicitors discussed herein.

(b) Co-express *Rx* directly with an appropriate elicitor (e.g. PVX CP from an avirulent strain). This approach has already been demonstrated by the inventors in the course of the isolation of the gene (to demonstrate complementation of the Rx trait). It was noted, however, that in certain cases in which both genes are constitutively co-expressed, an HR was produced within 48 hours. Thus if it desired to avoid a widespread HR, one of the following approaches may be

preferred:

(c) Co-express *Rx* and an elicitor gene, the transcription or translation of which is suppressed by the activation of Rx. This would recouple Rx to its elicitor, and better mimic the natural response to PVX infection which results in broad specificity silencing. This could be achieved, for instance, by expressing the PVX CP within an amplicon construct such as that described in Angell & Ballcombe (1997) The EMBO Journal 16,12:3675-3684. In this embodiment the suppression of the PVX/CP accumulation may preempt the HR.

(d) Co-express *Rx* with an elicitor gene, the translation of which is only switched on in the presence of pathogen(s). This could be achieved, for instance, by co-expressing Rx with a post-transcriptionally silenced elicitor (e.g. PVX CP) which will be unsilenced, possibly locally, in the presence of pathogen. In the absence of pathogen there would be no activation of the Rx mechanism. In the presence of pathogens which suppress gene silencing (which may, for instance be PVY or related viruses - see Pruss et al., 1997) the PVX CP would be unsilenced in the region infiltrated by PVY, and the HR would be limited to that region.

(e) Co-express *Rx* with an elicitor gene, whereby one or both are inactivated, and reactivate the gene(s) in a variegated manner, such that the HR is limited only to certain sectors of the plant (e.g. somatically defined sectors) but whereas the defensive response extends beyond these sectors. This could be achieved, for instance, by analogy with the methods disclosed in WO 95/31564 (GATSBY CHARITABLE FOUNDATION),

wherein, following a backcross between a plant carrying a transposon tagged resistance gene (in that case cf-9) plus intact elicitor (Avr-9) and a plant carrying an activator transposase, the progeny exhibited a somatic reactivation of the cf-9, leading to a localised necrotic response but widespread resistance.

In addition to the vectors and constructs above, the present invention also provides methods comprising introduction of the *Rx* constructs discussed above (such as vectors) into a host cell and/or induction of expression of a construct within a plant cell, by application of a suitable stimulus, an effective exogenous inducer.

The vectors described above may be introduced into hosts by any appropriate method e.g. conjugation, mobilisation, transformation, transfection, transduction or electroporation, as described in further detail below.

In a further aspect of the invention, there is disclosed a host cell containing nucleic acid or a vector according to the present invention, especially a plant or a microbial cell.

Thus DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green *et al.* (1987) *Plant Tissue and Cell Culture*, Academic Press),

electroporation (EP 290395, WO 8706614 Gelvin Debeyser) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman *et al. Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Recently, there has been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (Toriyama, et al. (1988) *Bio/Technology* 6, 1072-1074; Zhang, *et al.* (1988) *Plant Cell Rep.* 7, 379-384; Zhang, *et al.* (1988) *Theor Appl Genet* 76, 835-840; Shimamoto, *et al.* (1989) *Nature* 338, 274-276; Datta, *et al.* (1990) *Bio/Technology* 8, 736-740; Christou, *et al.* (1991) *Bio/Technology* 9, 957-962; Peng, *et al.* (1991) International Rice Research Institute, Manila, Philippines 563-574; Cao, *et al.* (1992) *Plant Cell Rep.* 11, 585-591; Li, *et al.* (1993) *Plant Cell Rep.* 12, 250-255; Rathore, *et al.* (1993) *Plant Molecular Biology* 21, 871-884; Fromm, *et al.* (1990) *Bio/Technology* 8, 833-839; Gordon-Kamm, *et al.* (1990) *Plant Cell* 2, 603-618; D'Halluin, *et al.* (1992) *Plant Cell* 4, 1495-1505; Walters, *et al.* (1992) *Plant Molecular Biology* 18, 189-200; Koziel, *et al.* (1993) *Biotechnology* 11, 194-200; Vasil, I. K. (1994) *Plant Molecular Biology* 25, 925-937; Weeks, *et al.* (1993) *Plant Physiology* 102, 1077-1084; Somers, *et al.* (1992) *Bio/Technology* 10, 1589-1594; WO92/14828). In particular, *Agrobacterium* mediated transformation is now emerging also as an highly efficient alternative transformation method in

monocots (Hiei *et al.* (1994) *The Plant Journal* 6, 271-282).

Microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

If desired, selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate.

Thus a further aspect of the present invention provides a method of transforming a plant cell involving introduction of a vector comprising a nucleic acid of the present invention (e.g. *Rx* or *Rx variant*) into a plant cell and

causing or allowing recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome.

The invention further encompasses a host cell transformed with nucleic acid or a vector according to the present invention, especially a plant or a microbial cell. In the transgenic plant cell (i.e. transgenic for the nucleic acid in question) the transgene may be on an extra-genomic vector or incorporated, preferably stably, into the genome. There may be more than one heterologous nucleotide sequence per haploid genome.

The term "heterologous" is used broadly in this aspect to indicate that the gene/sequence of nucleotides in question have been introduced into said cells of the plant or an ancestor thereof, using genetic engineering, i.e. by human intervention. A heterologous gene may be additional to a corresponding endogenous gene. Nucleic acid heterologous, or exogenous or foreign, to a plant cell may be non-naturally occurring in cells of that type, variety or species. Thus the heterologous nucleic acid may comprise a coding sequence of or derived from a particular type of plant cell or species or variety of plant, placed within the context of a plant cell of a different type or species or variety of plant.

Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., *Cell*

Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, *et al.* (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

Plants which include a plant cell according to the invention are also provided, along with any part or propagule thereof, seed, selfed or hybrid progeny and descendants. A plant according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of the plant or an ancestor thereof.

In addition to the regenerated plant, the present invention embraces all of the following: a clone of such a plant, seed, selfed or hybrid progeny and descendants (e.g. F1 and F2 descendants) and any part of any of these, such as cuttings, seed. The invention also provides a plant propagule from such a plant, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on.

As an alternative to the molecular-biology based methods of introducing Rx (or variants thereof) into plants, the sequences disclosed herein may be used to facilitate selection of plants into which it is desired to introduce the resistance trait using conventional plant breeding methods. Progeny from crosses which carry the gene may be readily identified by screening on the basis of the *Rx* sequence, particularly the *Rx* signature sequence.

The methods disclosed herein for identifying proximal markers to the *Rx* locus may be generally applicable to other genes found in clusters (e.g. plant derived resistance genes). Such methods are characterised in that they employ a step using low stringency PCR with non-degenerate primers which avoid conserved sequence motifs. The general approach may be summarised as follows:

(a) Prepare a population in which the gene of interest is segregating,

(b) Identify resistance gene homologue(s) linked to the locus of interest on the basis of highly conserved (resistance gene) motifs and highly degenerate primers (Leister *et al.*, 1996),

(c) Identify further markers corresponding to homologous genes, which are within the (resistance) locus and that are closer to the gene, using low stringency PCR with non-degenerate primers which avoid conserved sequence motifs,

(d) Use said further markers to identify a clone carrying the (resistance) gene of interest genomic library from a resistant plant, optionally in conjunction with transient assays for activity (Mindrinos et al (1994) or as described

above),

(e) Optionally, confirm the identity of the cloned gene on the basis of phenotype in transgenic plants.

The present invention also encompasses the expression product of any of the *Rx* or variant nucleic acid sequences disclosed above, and methods of making the expression product by expression from encoding nucleic acid therefor under suitable conditions, which may be in suitable host cells.

Antibodies may be raised to a purified Rx/variant polypeptide or peptide by any method known in the art (for an overview see e.g. "Immunology - 5th Edition" by Roitt, Brostoff, Male: Pub 1998 - Mosby Press, London).

Such antibodies, or fragments or derivatives thereof, can be used to bind Rx, or in the identification and/or isolation of proteins homologous to Rx (i.e. which share epitopes therewith), which in turn can provide the basis of an alternative method to those described above to isolate their encoding genes.

The invention further provides a method of influencing or affecting a resistance trait (preferably ER trait) in a plant, whereby the method includes the step of causing or allowing expression of a heterologous nucleic acid sequence as discussed above (e.g. *Rx* or *Rx variant*, in each case plus an optional elicitor) within cells of the plant.

As an alternative, it may be desirable to down-regulate Rx activity. This may be achieved, for instance used

antisense technology (which is reviewed in Bourque, (1995), *Plant Science* 105, 125-149, and Flavell, (1994) *PNAS USA* 91, 3490-3496). An alternative to anti-sense is to use a copy of all or part of the target gene inserted in sense, that is the same, orientation as the target gene, to achieve reduction in expression of the target gene by co-suppression. See, for example, van der Krol *et al.*, (1990) *The Plant Cell* 2, 291-299; Napoli *et al.*, (1990) *The Plant Cell* 2, 279-289; Zhang *et al.*, (1992) *The Plant Cell* 4, 1575-1588, and US-A-5,231,020.

Preferably, though, the invention provides a method which includes expressing Seq ID No 3 or a variant thereof within the cells of a plant (thereby producing the encoded polypeptide), following an earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof

Generally such a method may be used to introduce viral resistance into the plant whereby an Rx-mediated resistance (ER or HR) is triggered by contact with an appropriate viral elicitor or other initiator or inducer. Certain methodologies are set out in the Examples below. Broadly speaking the elicitor or other trigger may be encoded directly by the invading virus (such as the coat proteins of PVX or certain other Potex- and Carlaviruses). Alternatively it may be expressed by a separate construct or transgene which is itself triggered or upregulated by the viral infection. Additionally, in both of these cases, modification of the Rx (variant) sequence may allow triggering by a non-natural elicitor, if this is preferred.

The formats described above, to assess Rx or Rx-derivative function with respect to a putative or known elicitor, themselves form a further aspect of the present invention. In particular the methods, for establishing gene for gene compatibility between elicitor and resistance gene, are characterised in that they include the steps of:

(a) causing or permitting the co-expression in cell of *Rx* or an *Rx* derivative with the elicitor,

(b) observing said cell for an HR,

(c) correlating the result of the observation made in (b) with the specificity of the *Rx* or the *Rx* derivative for the elicitor.

Preferably the expression of the elicitor is decoupled from that of the *Rx* e.g. by use of a strong, constitutive promoter.

SEQUENCE ANNEXES

Annex I: shows the sequence derived from BAC77 (designated Seq ID No 1) and containing the *Rx* gene. Putative initiation codon (ATG) of *Rx* gene is given in bold and underlined. Two introns in the 3'-end of the *Rx* gene are underlined.

Annex II: shows a partial nucleotide sequence of the Rx cDNA including the Rx-coding nucleic acid sequence (designated Seq ID No 3).

FIGURES

Fig 1: shows the putative Rx polypeptide sequence

(designated Seq ID No 2). Regions corresponding to characterised domains are given in bold and underlined. Conserved aliphatic residues (L, V, I, F, M) in the LRR region are underlined. Two duplicated acidic fragments in the C-terminus of Rx are indicated by arrows.

Fig 2: shows a sequence alignment between Rx and products of four R genes encoding proteins containing putative leucine zipper/nucleotide binding site/leucine-rich (LZ-NBS-LRR) repeat motifs. The protein sequences aligned with DNASTAR software using the CLUSTAL method with PAM250 residue weight table. Highly conserved residues are indicated by black boxes. Dashes designate gaps introduced to improve the alignments. Abbreviations: Rx - Rx protein of the present invention; PRF fragment - C-terminal fragment (residues 841 - the end) of the *Lycopersicon esculentum* Prf protein (SPTREMBL accession Q96485); RPS2 - *Arabidopsis thaliana* Rps2 protein (PIR accession A54809); RPM1 - *A. thaliana* Rpm1 protein (PIR accession A57072); I2C-1 - *L. esculentum* resistance complex I2C-1 protein (SPTREMBL accession O24015; Ori et al (1997) Plant Cell, 9: 521-532.).

Fig 3: High resolution map of the *Rx* locus based on the Experiments disclosed herein (not drawn to scale).

The top panel shows a simplified genetic map of potato chromosome XII (denoted by a horizontal line) in which the area left of the arrow is reversed in the potato and tomato genetic maps (Tanksley *et al.*, 1992). Vertical lines indicate positions of previously mapped RFLP markers (Bendahmane *et al.*, 1997; Tanksley *et al.*, 1992). The filled rectangle denotes a genetic interval between markers GP34

and 218L, which is magnified in the panels below.

The middle panel shows the genetic map of the GP34–218L interval (denoted by a horizontal line). Positions of the RFLP marker GP34 and the AFLP markers IPM3, IPM4-a and IPM5 are indicated by vertical lines. The positions of BAC end-derived markers are indicated by arrows. The positions of markers enclosed in parentheses (*e.g.* 45L-b) have not been determined precisely. The numbers in square brackets indicate the numbers of S1 Cara individuals containing recombination events in each marker interval. The predicted position of *Rx* is indicated by '↔' symbol.

The bottom panel shows positions of BAC clones in GP34–218L interval. Each open rectangle represents one BAC insert DNA. Inside of each rectangle is the name of the BAC clone, the estimated insert size in kb (except for the BAC29) and a symbol 'L' or 'R' denoting the BAC ends that had been mapped relative to *Rx*.

Fig 4: Alignments of putative peptide sequences derived from *Rx*-linked markers.
(a) The aligned putative peptide sequence of the IPM4 marker with homologous region of Prf (Salmeron *et al.*, 1996), and (b) the putative peptide sequences of 73L marker aligned with homologous regions of Cf-2 and Cf-9 proteins (Dixon et al., 1996; Jones *et al.*, 1994). Sequences were aligned using BLAST (Altschul *et al.*, 1990). Identical residues are indicated in bold, similar residues are in black, and different residues are in grey.

Fig 5: Introgressed DNA in the vicinity of *Rx*.

The diagrams show chromosomal DNA in the IPM3—IPM5 interval from potato cv Cara and clone SH83 and from individuals in our S1 Cara mapping population (identified in left hand column). These plants were either *Rx* (R) or *rx* (S) genotypes and the chromosomal region introgressed from *S. tuberosum* spp. *andigena* is shown as a close rectangle. The *S. tuberosum* spp. *tuberosum* DNA is shown as a thin line. DNA markers used to map the introgressed DNA are identified over the vertical lines, and the region containing *Rx* is delineated by the horizontal arrow.

Fig 6: shows regions of the BAC77 clone which were used in transient assay experiments (A) and also a construct (PVX-TK) used in the experiments (B).

Fig 7: shows a sequence alignment between *Rx* (from *S. andigena*), *Rx*2 (from *S. acaule*) and other *Rx* homologues.
*Rx*1 indicates sequence from *Rx* from S. andigena.
Ac15 and Ac64 indicate two sequences from two independent colonies carrying *Rx*2 from *S. acaule* .
111h1 and 221h2 indicate two *Rx* homologous sequences from BAC111 and BAC221 respectively.
K39.hom indicate an *Rx* homologous sequence from the natural potato hybrid *S.* X *juzepczukii* carrying Rx_{juz}
(A) is a DNA sequence alignment,
(B) is a protein sequence alignment.

Fig 8: shows a comparison between the sequence of the coat protein of PVX and the coat protein of other viruses of the Potex- and Carlaviruses groups. As shown in the Figure, there are certain regions of sequence conservation within the coat proteins (solid black shade = residues that match

consensus exactly).

EXAMPLES

Example 1- Assessment of BAC library

The description below sets out how the present inventors cloned Rx.

A BAC library of 160000 clones from plant SC-781, which is a progeny of selfed cv Cara carrying *Rx* in the duplex condition (*Rx,Rx,rx,rx*), was prepared. Based on the average insert size of 100 kb in the BAC clones (data not shown), a haploid genome size of 900000 kb in potato (Arumuganathan and Early, 1991) and taking into account the presence of *Rx* in the duplex condition we estimated that there was more than a 99% probability that this library carried the DNA of the *Rx* (or any other) locus. The estimate was based on the formula: N=Ln (1-P)/Ln (1-I/G), where I is the size of the average cloned fragment in base pairs, and G is the size of the genome, in base pairs (Clark and Carbon, 1976). According to this formula, for any gene carried in the duplex condition, there would need to be ≤80000 BAC clones of 100 kb to have a 99% probability of representation in a library.

To confirm the genome representation in this potato BAC library we screened for the clones containing single copy co-dominant cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel, 1993) markers: GP34, PM3, CT99 and CT129 that are linked to *Rx* (Bendahmane *et al.*, 1997). In each instance we obtained between 1 and 8 positive clones

(data not shown). At least one clone identified with each marker tested contained an allele linked in *cis* to *Rx*. Therefore, the BAC library represents a large part of the potato genome and could be used to identify BAC clones spanning many regions of interest in the potato genome.

Screening of the potato BAC library with markers closely linked to and flanking Rx *locus*

Bendahmane *et al.*, 1997 discloses CAPS markers, IPM3 and IPM4, that flank *Rx* and span an interval of 0.23 centiMorgans (cM). Using the IPM3 marker to screen the BAC library we identified three BAC clones: BAC167, BAC191 and BAC117, with potato DNA inserts ranging from 100 to 120 kb. *Dde*I digestion of the IPM3 DNA in these BACs and in potato DNA samples revealed that BAC117 carried the IPM3 allele that was linked in *cis* to *Rx*. The other two BACs, BAC167 and BAC191, contained alleles from a corresponding region of the *rx* chromosomes.

To identify the relative genome positions of these BACs we designed pairs of PCR primers based on the sequence of the right and left ends of the insert (see Experimental procedures). PCR tests using the BAC DNAs as templates showed that these BACs overlapped in the order BAC167, BAC117, BAC191, *Rx*. The 191L marker was separated from *Rx* by only a single chromosomal recombination event (in plant #1146) in a mapping population of 1720 plants. In the same population, 117L and IPM3 markers were separated from *Rx* by two and three recombination events respectively whereas the GP34 marker, present in BAC167, was separated from *Rx* by thirteen recombinations (Figure 3). The BAC library did not

contain additional BACs extending further towards *Rx* from the 191L marker.

Characterization of the BAC clones linked to the IPM4 CAPS marker

The IPM4 marker was previously mapped at 0.06 cM from *Rx* on the side away from IPM3 (Bendahmane *et al.*, 1997). Screening of the BAC library with IPM4 identified two clones: BAC73 and BAC111, with inserts of ~70 kb each. The *Taq*I digestion of the IPM4 CAPS marker in these clones suggested that BAC111 was linked in *cis* to the *Rx* locus whereas BAC73 carries DNA insert from the *rx* chromosome (data not shown).

To determine the relative genome position of BAC111 and BAC73 we performed PCR tests using end sequence primers of these BAC clones. These are shown in Table 1:

Table 1. PCR-based markers linked to *Rx* locus.

MARKER	PRIMERS	PCR conditions	Restriction enzyme
117L	5'- CCTAGCGTAGAGCGGTGTATCCA 5'-GTAGACATTTAATAATTCGTCGATAC	94°C, 15 sec 57°C, 20 sec x 35 cycles 72°C, 120 sec	*Rsa*I
191L	5'- ACAAATTGTATAATTATAGTGATACG 5'- CAAGACATTAATTAACCAAACAATGG	94°C, 15 sec 50°C, 15 sec x 35 cycles 72°C, 120 sec	*Eco*RI

77L	5'- GCTTCTAAACTCTAAATTCAGATTC 5'- CATGTGCGGACTCGTTCTTTTGTAG	94°C, 15 sec 64°C, 15 sec x 35 cycles 72°C, 60 sec	*AluI*
IPM4	5'- GTACTGGAGAGCTAGTAGTGATCA 5'- GAACACCTTAACTACACGCTGCAGG	94°C, 15 sec 62°C, 15 sec x 35 cycles 72°C, 90 sec	*TaqI*
77R*	5'- GAAAGACAATTCCAGTGTGATGCG 5'- CAGGTAAGCCTCCTCATAACATGC	94°C, 15 sec 66°C, 15 sec x 35 cycles 72°C, 60sec	
45L*	5'- GGAGTCAATGCAGGGTCTATGGAA 5'- CTCATTTGACACTTCTCGAACACA	94°C, 15 sec 62°C, 15 sec x 35 cycles 72°C, 50 sec	
221R*	5'- GCTTACATTTGCTCGAAGAAGCCAC 5'- CCTTAATAATCAATAGATTCAACTCG	94°C, 15 sec 60°C, 15 sec x 35 cycles 72°C, 60sec	
111L*	5'- CCGAGTTTGCTCGATTCCGAGTTTT 5'- CTAAGGGATCCACTAGTCTAATTTG	94°C, 15 sec 62°C, 15 sec x 35 cycles 72°C, 60 sec	
111R*	5'- CCACTGTGTAAGGGTCAACTATAGTC 5'- GAGATGAAGATTTTCTTGTCTGATGG	94°C, 15 sec 65°C, 15 sec x 35 cycles 72°C, 90 sec	
Cos98	5'- GCGAGATAAAGACATGATAAGAGAT 5'- GAATTTGGAATGAAGATCAACAGTC	94°C, 15 sec 62°C, 15 sec x 35 cycles 72°C, 60 sec	*AluI*

73L	5'- CATTTCCTGAATTGCTTCCGACTTC 5'- CCATGAAAATTGTTATCACTGAGGTC	94°C, 15 sec 60°C, 15 sec x 35 cycles 72°C, 50 sec	*AluI*
218R	5'- GATTACAGTTGTGAATTAGTTCGGTA 5'-GCAACAGATATATTCCACTTACCATTC	94°C, 15 sec 60°C, 15 sec x 35 cycles 72°C, 90 sec	*AluI*
218L *	5'- CTTAACAAACCTATCATATTGGCCAT 5'- AGCTTCACATTGAACCAGAGGCCT	94°C, 15 sec 62°C, 15 sec x 35 cycles 72°C, 90 sec	

* These markers are allele-specific (i.e. marker primers are able to amplify only an allele linked in *cis* to *Rx*). These markers do not require restriction enzyme digestion.

These tests suggested that BAC73 overlaps with BAC111 and that 73L and 111L represent opposite ends of this set of overlapping BACs. Both, 73L and 111L, co-segregated with IPM4. In our mapping population of 1720 individuals these markers were separated from *Rx* by one recombination event (in individual #761) and it was not possible to determine directly which of these markers was physically closer to *Rx*. Hence, to orientate these BACs relative to *Rx* we screened the BAC library with CAPS markers 111L and 73L. We also screened the BAC library with the IPM5 CAPS marker which is on the same side of *Rx* as IPM4, but further from *Rx* (Bendahmane *et al.*, 1997). It was hoped that BACs containing IPM5 would allow us to orientate the 111L and 73L markers relative to *Rx* (Figure 3).

These analyses identified BAC218, carrying an allele of IPM5 identified by *Pst*I digestion as being linked in *cis* to *Rx* (data not shown). The end sequences of BAC218 insert DNA were converted into the CAPS markers, 218L and 218R, and mapped genetically to the recombination events between GP34 and IPM5. Marker 218L was positioned at 8 recombination events (0.48 cM) from *Rx*, between IPM5 and CT129. The 218R marker was positioned between IPM4 and IPM5 at five recombination events (0.30 cM) from *Rx*. We also identified a single BAC pool #29 which contains three markers: 218R, 73L and 111R. CAPS analysis revealed that each of these markers in the BAC pool #29 is represented by the allele linked in *cis* to *Rx* (data not shown). Hence, we conclude that BAC pool #29 contains a single BAC clone, BAC29, with DNA insert linked in cis to *Rx*. Therefore, BAC29 provided a link between BAC218 and the IPM4 BAC contig and allowed us to orientate the markers from the IPM4 contig in the following order: *Rx*, 111L, IPM4, 73L (Figure 3).

By screening the BAC library with 111L allele-specific primers we identified BAC221 which carries an insert DNA of 50 kb and is linked in *cis* to *Rx* (data not shown). The left end sequence of BAC221 is located inside of BAC111 whereas the right end sequence of BAC221 extends further towards *Rx* (Figure 3). However the marker 221R co-segregated with IPM4 (Figure 3) in our mapping population and was separated from *Rx* by the recombination event in plant #761.

To extend the IPM4 contig further towards *Rx* we screened the BAC library with 221R allele-specific primers and identified BAC45 which has an insert DNA of 50 kb and is linked in *cis* to *Rx* (data not shown). The right end sequence of BAC45 is

located inside of BAC221, whereas the left end sequence of BAC45, 45L, extends further towards *Rx* (Figure 3).

However, BAC45 does not contain *Rx* as the CAPS marker 45L is genetically separated from *Rx* by the recombination event in plant #761. Additional PCR screening of the BAC library with the 45L marker failed to identify any new BAC clones therefore leaving a gap between the IPM3 and IPM4 BAC contigs (Figure 3).

Analysis and utilization of duplicated DNA sequences for mapping of Rx

Comparison using the BLAST search (Altschul *et al.*, 1990) of the translated version of CAPS marker sequences with the PDB, SWISS-PROT, PIR(R), GenPept, and GenPept databases revealed that several BAC-derived markers from IPM4 contig are similar to cloned disease resistance genes (Figure 8). The marker IPM4 was similar to the tomato *Prf* gene required for the resistance to *Pseudomonas syringae* pv. *tomato* (Salmeron *et al.*, 1996), and the marker 73L showed a significant degree of homology to the *Cf-2* and *Cf-9* resistance genes of tomato (Dixon *et al.*, 1996; Jones *et al.*, 1994) (Figure 4). These data indicated that *Rx* may be in a cluster of disease resistance-like genes.

Disease resistance loci in plants are often highly complex with small families of resistance genes clustered within several dozen kilobases (Ellis *et al.*, 1995; Hulbert and Bennetzen, 1991; Jones *et al.*, 1994; Martin *et al.*, 1993; Witham *et al.*, 1994). These clusters are due to structural duplications of different sizes within the disease

resistance loci which may reflect a mechanism of evolution of disease resistance loci (Anderson *et al.*, 1996; Song *et al.*, 1997). Taking into account our results, we tested for the presence of the duplicated sequences related to CAPS markers from the vicinity of *Rx* (IPM3–IPM5 interval). In these tests we used pools of DNA from 20 resistant plants (R pool) and 20 susceptible plants (S pool) and the individual BAC clones from the IPM4 contig as templates for PCR amplifications. The primer annealing temperature in PCR reactions was 5 to 10°C lower than in conditions originally developed for each CAPS primer pair (Table 1) so that there would be amplification of the original marker and related homologues. The PCR products obtained with a number of tested CAPS primer pairs were the same size as the products produced under high stringency conditions (data not shown). However, digestion of these low stringency PCR products with either *Taq*I, *Alu*I or *Dde*I restriction enzymes revealed several new DNA fragments that were not identified previously. These included fragments that were nonpolymorphic as well as fragments polymorphic between the R and S pools. However, we allocated most of these new fragments to BAC clones from the *Rx* locus.

The low stringency PCR products obtained with 45L primers and digested with *Dde*I produced two new fragments from the R pool DNA template in addition to those produced from the S pool and from BAC45. These fragments were also obtained after *Dde*I digestion of the BAC111-derived PCR product (data not shown). The previously identified 45L marker is referred to as 45L-a and the newly identified locus in BAC111 as 45L-b (Figure 3).

The Cos98 primers were based on a sequence in the BAC221 insert DNA (A.B., K.K. and D.C.B., unpublished data). They primed a PCR product from potato DNA of *Rx* plants that could be identified by *Alu*I digestion as the original locus in BAC221 (this locus is designated as Cos98-b). Under the low stringency conditions they also identified alleles or new loci present in the adjacent BAC45 (Cos98-a) and BAC111 (Cos98-c) (Figure 3).

Digestion of the low stringency IPM4 products from the R pool with *Taq*I identified the original IPM4 locus (IPM4-a) in BAC111. There were also IPM4 restriction fragments that had not previously been detected (data not shown). One of these fragments was nonpolymorphic in the R and S pools. This fragment must have originated from BAC221 as the *Taq*I restriction fragment of similar size was also detectable after digestion of IPM4-b allele derived from this BAC (data not shown). However, second new DNA fragment was polymorphic between R and S pools and was not detected after digestion of either IMP4-a or IPM4-b alleles derived from BAC111 and BAC221 correspondingly (data not shown). This fragment segregated with *Rx* in all plants of our mapping population, including plant #761 and others with recombination events between GP34 and IPM5 (data not shown). This new IPM4 marker allele co-segregating with *Rx* was designated IPM4-c.

The potato BAC library was screened by PCR with IPM4 primers using conditions for detection of the IPM4-c allele. One new BAC clone with DNA insert of ~50 kb, BAC77, carrying the IPM4-c locus was identified (data not shown). The end fragments of BAC77 DNA insert were cloned, sequenced and converted into the CAPS markers, 77L and 77R. The marker 77L

co-segregated with both IPM4-c and *Rx* whereas 77R was separated from *Rx* by one recombination event in the recombinant individual #761 (based on analysis of 1720 segregants; see Figure 3 and Figure 5).

BAC77 spans Rx

To ascertain whether BAC77 spans *Rx*, an additional 1438 individuals from the S1 Cara population were screened with markers 77L, IPM4-c and the flanking markers 191L and 77R (data not shown). There were no plants with recombination events between *Rx* and markers 77L and/or IPM4-c. However, three individuals B#232, B#934 and B#1393 were found with recombination events in the 191L—77L interval. All of these plants contained *rx*-linked alleles of 191L but were resistant to PVX and contained the *Rx*-linked allele of 77L. These data suggest a tight linkage between *Rx* and the marker 77L (Figure 3) but did not allow *Rx* to be conclusively positioned in the 191L—77R interval.

Further data supporting the conclusion that BAC77 spanned *Rx* was provided by 'introgression mapping' experiments carried out on *Globodera pallida* resistance gene *Gpa2* (data not shown). Like *Rx*, this gene resides on the short arm of the potato chromosome XII (Rouppe van der Voort *et al.*, 1997). Both *Gpa2* and *Rx* were introgressed into cultivars Cara and Amaryl from the *Solanum tuberosum* ssp. *andigena* breeding line CPC1673. A dihaploid potato clone SH83-92-488 (referred to hereafter as 'SH83') derived from the cultivar Amaryl carries both *Rx* and *Gpa2*. To map the CPC1673-introgressed DNA in cv Cara, cv Amaryl and clone SH83 the DNA of these

potato genotypes was subjected to PCR using a number of CAPS markers in the GP34—IPM5 genetic interval. This analysis provided evidence that all these potato genotypes contain DNA introgressions from the line CPC1673 within the GP34—IPM5 interval. However, the size of introgressed DNA segments varied between these genotypes (Figure 5). Line CPC1673 and cv Cara contain *Rx*-linked alleles of each CAPS marker within the GP34—IPM5 interval. However, in cv Amaryl and clone SH83, the alleles originated from the CPC1673 clone and linked in *cis* to *Rx* could be identified only for markers in the IPM4-c—IPM5 interval (Figures 5 and 3, and data not shown). Therefore, the introgression mapping data together with the recombination data strongly suggest that *Rx* is located between markers 77L and 77R (Figure 5) in the vicinity of IPM4-c.

Example 2 - Confirmation in plant cells that BAC77 contained coding sequence for Rx

Confirmation was carried out by transient assays based on the use of BAC77 DNA, an appropriate initiator (PVX or PVX CP) and a reporter gene.

The BAC77 DNA was combined in a single plasmid, with a PVX-GUS cDNA, under the control of a CaMV 35S promoter. In the experiments, an avirulent (PVX-TK) or a virulent (PVX-KR) strain of PVX under the control of 35S promoter was cloned into SrfI unique site of BAC77 to obtain BAC77(PVX-TK) and BAC77(PVX-KR) respectively. To monitor virus replication the GUS gene was expressed from duplicated PVX coat protein promoter - expression of GUS requires efficient accumulation of PVX-GUS in infected cells. A mutation

within the PVX ORF (encoding the 25-kD movement protein) ensured that GUS expression would only report virus replication in cells also expressing the candidate *Rx*.

The BAC77 regions used in the assays, and an Example construct (PVX-TK) are shown in Fig 6(A) and (B) respectively.

It was predicted that because BAC77 carries *Rx* function, bombardment of *N. benthamiana* leaves with gold particles coated with DNA from BAC77(PVX-TK) or BAC77(PVX-KR) would lead to GUS expression only with the latter.

The BAC77/PVX-GUS plasmid was introduced into tobacco cells and susceptible potato cells by biolistic transformation using coated gold particles. Results showed that there were approximately ten fold more GUS+ cells if the PVX-GUS was based on an Rx-breaking strain of PVX-KR than with a common, Rx-avirulent, strain of PVX-TK. This is consistent with activation of Rx resistance by expression of the avirulent PVX-TK virus, leading to suppression of the PVX-GUS activity. The result confirmed the gene-for-gene relationship between BAC77 and virulent inducer (PVX-KR).

This system was used for fine mapping of *Rx* locus within the 50 kb BAC77. Five sub-clones of BAC77 were prepared using overlapping DNA fragments. These were cloned into the 'virulent' and 'avirulent' plasmids and tested as above. Results showed that functional *Rx* was present both in an 18 kb fragment of DNA in BAC9 (designated FR9) and, within this, in a 11 kb PvuII DNA fragment present in BAC6.

The sequence of these regions (see Annex I) indicated that there is a single gene in the regions of BAC77 that is linked to the Rx phenotype. This gene was accordingly designated *Rx* and its product as Rx.

Example 3 - Rx is active in transient expression studies in plants which are not PVX hosts

Rx/PVX-GUS constructs were assembled in which *Rx* was under control of the 35S promoter. These constructs were bombarded into leaves of *Arabidopsis* plants. As with the experiments described above there were approximately ten fold more GUS+ cells if the PVX-GUS was based on an Rx-breaking strain of PVX than with a common strain of PVX. This result shows that the cloned Rx was active in *Arabidopsis* which is not a normal host of PVX.

Example 4 - Transiently expressed Rx can suppress PVX

The Rx/PVX GUS constructs were also introduced into *N.tabacum, N.benthamiana, L. esculentum* and potato cells using Agrobacterium-mediated transformation. Briefly, the BAC77 DNAs were transferred into a binary Ti plasmid vector in Agrobacterium tumefaciens. The Agrobacterium cells were then infiltrated into leaves of the test plants. The Ti plasmid constructs had different regions of BAC77 and in some instances, as in the biolistic experiments, included PVX-GUS cDNA from common strain or Rx-breaking strain PVX. When the PVX-GUS in these constructs was from a resistance breaking strain of PVX there was abundant GUS expression in all samples tested, indicating that the infiltration method was an efficient procedure for inoculation of PVX. In

contrast, if the PVX-GUS was from a common strain of PVX and if the BAC77 DNA was from the region most closely linked to *Rx*, there was less GUS. This result indicated that the transient expression of Rx in the cells of the infiltrated part of the leaf suppressed PVX accumulation in the same way as the native Rx gene.

Example 5 - Stable transformation of Rx and ER in susceptible potato plants

In order to confirm the earlier transient-expression experiments, transgenic plants of *Solanum tuberosum* cultivar Maris Bard with FR9 were produced.

Rx in plants

The plant transformation was carried out using SLJ7292 binary vector (Jones *et al*, 1992). The larger 18 kb FR9 Rx fragment was used in order to ensure that the sequence encoded all of the required regulatory sequence.

Infectivity assays were performed using mechanical inoculation of PVX strains. Seven of eight independent transgenic lines carrying the FR9 insert were resistant to *Rx*-avirulent PVX-TK and susceptible to *Rx*-virulent PVX-KR. This resistance was manifested as the absence of PVX-TK symptoms and complete lack of PVX-TK accumulation in inoculated and systemic leaves. This resistance reaction is phenotypically similar to that observed in the wild type potato cultivar CARA.

Rx in protoplasts

PVX accumulation in protoplasts from two of the transgenic lines, M4 and M7, was assessed. For comparison protoplasts from Cara (*Rx*) and Maris Bard (*rx*) were also tested. The protoplasts were inoculated with PVX-TK and PVX-KR and the RNA was sampled 24 hr after inoculation. Gel blot analysis confirmed that there was no accumulation of PVX-TK in protoplasts of cultivar Cara and in the two transgenic lines. By contrast, PVX-TK did accumulate in Maris Bard. PVX-KR accumulated in all lines. This confirmed that transgenic *Rx*-mediated resistance is consistent with the *Rx*-phenotype in Cara.

Example 6 - The *Rx* transgene also confers resistance in heterologous plants species

The transient-expression experiments described in earlier examples established *Rx* function in *Arabidopsis, L. esculentum, N. tabacum* and *N. benthamiana*. To demonstrate that actual ER could be introduced into at least some of these species, transgenic plants were produced using the FR9 Rx DNA.

Plants of 18 transgenic lines (six each of *L. esculentum, N. tabacum* and *N. benthamiana*) were rub inoculated with *Rx*-virulent and *Rx*-avirulent strains of PVX. RNA analysis of inoculated and systemic leaves two weeks later revealed that PVX accumulated only when the breaking strain was used as an inoculum.

In order to establish whether of not any HR response (and corresponding cell death) was occurring in the transgenic

plant, a more rigorous test (graft inoculation) was used. All graft inoculations described below were reproduced in two independent experiments. Scions (upper part) of *N. benthamiana* carrying Rx were grafted to a stock (lower part) of a nontransgenic *N. benthamiana* that had been preinoculated with PVX-TK (ten independent grafts). As a control, we used *N. benthamiana* plants carrying the TMV resistance gene *N*. These plants were resistant against a TMV vector expressing the jellyfish green fluorescence protein (TMV-GFP). This N-mediated resistance was manifested at 4 days after inoculation as localized HR in TMV-GFP inoculated leaves (data not shown). We never observed systemic symptoms in these plants indicating that the N mediated resistance restricted TMV to the inoculated leaf. In TMV-GFP graft inoculated plants (ten independent grafts) from 10 days after grafting, the HR was manifested as a systemic HR. Eventually this HR spread to cause death of the scion. The spreading HR was not observed in TMV-GFP graft inoculated scions that did not carry N. These controls demonstrated how the HR of graft inoculated plants was no longer restricted to local necrotic lesions as occurs in rub inoculated leaves. Thus, the graft inoculation exaggerates the HR.

In the graft inoculated scions of a nontransgenic *N. benthamiana,* there were high levels and mosaic symptoms of PVX-TK, indicating that PVX could cross the graft union. However, the scions of transgenic Rx plants were symptomless, even after several weeks. There was no evidence of HR even by examination of leaves of the Rx scions under a dissecting microscope or after trypan blue staining (Parker et al., 1993) for cell death (data not shown), and there was no accumulation of PVX-TK.

We also carried out a double graft experiment in which a scion of *N. benthamiana* (transgenic Rx genotype) was grafted between a healthy scion and a preinoculated with PVX-TK stock of nontransgenic *N. benthamiana*. We wanted to determine whether PVX-TK was able to cross a graft union and to pass through the vascular tissue of an *Rx* genotype plant. In ten independent double grafts RNA gel blot analysis showed PVX-TK accumulation in the inoculated stock and in the upper (nontransgenic) scion but not in the intermediate scion. From these results, we conclude that Rx-mediated resistance did not suppress PVX-TK translocation through the phloem. These results also confirm that, in the single grafted plants, there would have been translocation of PVX-TK into the transgenic Rx scion and the potential for activation of an *Rx*-mediated HR. From the absence of an HR in these graft inoculated plants, we conclude that there is no cell death associated with Rx-mediated resistance in *Nicotiana* species.

Example 7 - Over-expression of Rx causes cell death

One of the common features of a number of R genes in plants (and apoptotic genes in animals) is a conserved structure that is characterized by a series of motifs that include the nucleotide binding site (NBS), GLPL, CFLY, and the MHD motifs (van der Biezen and Jones, 1998). In *C. elegans* it was shown that over-expression of CED-4, an apoptotic gene of similar structure to plant R genes, leads to cell death. Similarly, the functional homologue of CED-4 in human, Apaf-1, lead to cell death in an over-expression experiment (Perkins et al, 1998).

To test whether plant R genes can also lead to cell death when over-expressed in plant cell, we expressed Rx cDNA under the control of the CaMV 35S constitutive promoter in *N. tabacum*. As control we prepared a second construct in which Rx cDNA was expressed from its weak wild type promoter. To deliver these Rx constructs into plants, they were introduced into binary pBin19 plasmid vector (Bevan, 1984) and transformed into *A. tumefaciens*. The *A. tumefaciens* cultures were then infiltrated into leaves of wild type *N. tabacum* via agroinfiltration (see general methods below).

When constructs encoding Rx under the 35S promoter (pBIN35-Rx) were agroinfiltrated into *N. tabacum* HR was observed. The necrosis first appeared within 48 hr post-infiltration and caused complete death of the infiltrated region by 72 hr. In contrast when Rx was expressed from its native weak promoter (pB1-Rx) the elicitation of the HR was dependent on the elicitor (PVX-CP).

Example 8 - Co-expression of Rx and the PVX-CP: relationship between ER and HR

In further experiments employing the agroinfiltration procedure and the binary pBin19 plasmid vector, the constructs also had regions of BAC77 but, instead of the PVX-GUS insert there was a 35S-PVX coat protein (CP) construct. This uncoupled the CP expression from PVX replication.

Infiltration of these constructs, carrying the cloned Rx, into *Rx* transgenic *N. Benthamiana* (line B18) leaves produced

no visible effect within 3d if the coat protein construct was derived from the resistance breaking strain PVX-KR, or non-transgenic plants were used. However if the coat protein construct was from the common strain PVX-TK there was a clear hypersensitive response produced within 48 hr, and complete cell death of the infiltrated region by 72 hr.

Other experiments with *Rx*-transgenic potatoes, and non-transgenic Cara (Rx) potatoes confirmed these results. This experiment confirms that there is a potential for *Rx*-mediated HR. However this potential is not realized when the PVX CP is expressed from the PVX genome during the viral infection cycle.

To further explore the relationship of ER and HR, we analyzed the effect of Rx-mediated resistance on the N gene-mediated, HR type resistance against TMV. These experiments employed tobacco plants carrying the N gene either alone or in combination with transgenic Rx. The plants were challenged with recombinant isolates of TMV expressing the CP gene from either the Rx-virulent (PVX-KR) or the avirulent (PVX-TK) strain of PVX (Bendahmane et al., 1995). The TMV-TK construct encodes the elicitors of both N- and Rx-mediated resistance (Padgett and Beachy, 1993; Padgett et al., 1997), whereas TMV-KR does not encode the elicitor of Rx-mediated resistance. Transcripts of the TMVTK and TMV-KR cDNAs were initially inoculated onto N. *benthamiana* and sap extracts were produced from the inoculated leaves at 8 days after inoculation. Tobacco plants carrying the N gene either alone or in combination with Rx were inoculated with these extracts containing high concentration of virus, without dilution. After 3 to 4 days the TMV-KR and TMV-TK induced HR

on N. *tabacum* expressing the N gene. There was also an HR when TMV-KR was inoculated onto plants of the N, Rx-genotype. However, when these plants were inoculated with TMV-TK there was no HR. These data indicate that the Rx--mediated ER was activated prior to the N-mediated resistance and, therefore, that extreme resistance is epistatic to an HR.

Conclusions from Examples 1 to 8

Isolation of Rx

The above Examples demonstrate the isolation of a BAC clone from potato that, according to genetic mapping criteria, spans the *Rx* locus in cv Cara. This was achieved notwithstanding a considerable variation in the frequency of recombination in the region of DNA introgressed into Cara from *S. tuberosum* ssp. *andigena*. In certain regions, including the region containing *Rx* from 77L to 73R, recombination was extremely rare. Thus in our mapping populations of 3150 progeny we detected only 2 recombination events in that interval of more then 170 kb.

Initially the presence of repeated DNA sequences around the *Rx* locus complicated characterization of BAC clones and construction of BAC contigs. For instance we isolated several BAC clones from our library that were not from the *Rx* chromosome although they had been initially screened with allele-specific PCR or CAPS markers (A.B., K.K. and D.C.B., unpublished). We now know that this anomaly is due to differential PCR in genomic and cloned DNA. Repeated DNA loci that do not get PCR-amplified from a genomic DNA

template may get amplified from cloned DNA because the primer-complementary sequences are more abundant in the cloned DNA than in genomic DNA and because there is no potential for competition for the primers between similar loci.

The complications associated with repeated sequences were compounded by the tetraploid nature of the potato genome and the high degree of polymorphism in the constituent genomes. At some loci there were four different alleles (A.B., K.K. and D.C.B., unpublished). There may be an additional complication with the *Rx* locus in that it is duplicated on chromosomes V and XII (Ritter *et al.*, 1991). However, despite these complications, we were able to actually exploit the presence of repeated sequences in and around the *Rx* locus in isolating the sequence itself.

Rx and the HR response

In Example 7, overexpression of the Rx gene led to cell death in susceptible species. Thus the modulation of the level of Rx expression may be used as a strategy to engineer disease resistance into plants. For example, *in planta*, overexpression of the *Rx* gene in susceptible species may be advantageous because:

(i) it could give rise to disease resistance against viruses related to PVX isolates which elicit weakly *Rx*-mediated resistance in wild type *Rx*-plant.

(ii) it could give rise to spontaneous induction of cell death in some cells of the leaves and thus lead to systemic acquired resistance against different pathogens. Such an approach has been demonstrated by the HR which resulted from

expressing Rx cDNA under a 35S promoter in an agroinfiltration assay in tobacco.

Effect of the elicitor on Rx and the HR response

Our interpretation of the results in Example 8 is based on a "hair trigger" hypothesis. We envisage that in PVX infected cells, and in the experiments described in the Examples above, the production of PVX CP from the viral genome would have activated Rx-mediated resistance at an early stage in the infection cycle because Rx is so sensitive to the presence of the coat protein elicitor. Virus accumulation would have been arrested before CP accumulation had reached a high level. Other work (data not shown) has confirmed that the induction of the resistance mechanism by CP does not require *de novo* transcription, and that the *Rx*-mediated resistance includes a mechanism that prevents growth or differentiation in cells.

In contrast, Rx mediated resistance would have no effect on elicitor production in cells infiltrated with the 35S CP construct. There would be continuous, decoupled, activation of the Rx mediated resistance rather as if the "hair trigger" of a repeating gun was held in place with a rubber band. This continuous activation of the resistance mechanism would expose secondary resistance responses (eg HR induced necrosis) that would not normally be evident in resistant plants after inoculation owing to the effectiveness of the primary ER.

Because *Rx* shares sequence similarity to many disease resistance genes, it is likely that variations of this model

are relevant to other disease resistance interactions in plants.

Example 9 - Cloning and sequencing Rx homologues and paralogues

Cloning and sequencing of Rx2.

There are two *Rx* loci mapped in potato conferring strain-specific resistance to PVX (Cockerham, 1970). These loci are on chromosome V (*Rx2*) and chromosome XII (*Rx1*) (Ritter et al, 1992; Bendahmane et al, 1997) but are functionally identical: both confer extreme resistance which is only overcome by the same natural or mutant isolates of PVX (Querci et al., 1995). *Rx1* originates from *S andigena* and is the same as *Rx* in potato cultivar CARA. *Rx2* originates from *S acaule*. In this section we describe the isolation of *Rx2* using primers based on *Rx* sequence (cloned from potato cultivar CARA) following the strategy described earlier. The basic scheme was as follows:

I-Production of *Rx2* segregating population.
II-PCR amplification of *Rx* homologues using primers flanking *Rx* gene and using DNA from bulked segregant as template.
III-Cloning of the PCR products in BIN19 binary vector.
IV-Screening for *Rx2* function using agroinfiltration transient assay described above.
V-Mapping of the candidate *Rx2* on potato genome.
VI-sequence analysis.
VII-Transgenic expression of *Rx2* in *N. benthamiana* and *N. tabacum*.

The *S. Acaule* homologue was cloned and sequenced as described in more detail in the Methods section below. The sequence is shown in Fig 7. *Rx*1 indicates sequence from *Rx* from S. andigena. Ac15 and Ac64 indicate two sequences from two independent colonies carrying *Rx*2 from *S. acaule* which are virtually identical.

The *Rx2* paralogue contains acidic and amide motifs in the C-terminal domains which are also present in the Rx gene and other paralogues on potato chromosome V as well as on chromosome XII. The *Rx2* paralogue is functionally identical to, but independent of, the *Rx* gene described in the Examples above.

Fig 7 also shows sequences 111h1 and 221h2; these are two *Rx* homologous sequences from BAC111 and BAC221 respectively.

Cloning and sequencing of extreme resistance genes from different potato species.

There are reports of extreme resistance to PVX not only in *S. andigena* and *S. acaule* but also in other potato species. These examples include Rx_{vrn} (*S. vernei*), Rx_{juz} (S. X *juzepczukii*, natural hybrid), Rx_{cur} (*S. X curtilobum*, natural hybrid), Rx_{cha} (S. X *chaucha*, natural hybrid) and Rx_{suc} (*S. Sucrense*). The isolation of Rx_{vrn}, Rx_{juz}, Rx_{cur}, Rx_{cha} and Rx_{suc} is achieved using methods analogous to those described above for the cloning of *Rx*2 from *S. acaule*. Fig 7 shows K39.hom, which indicates an *Rx* homologous sequence from the natural potato hybrid *S.* X *juzepczukii* carrying Rx_{juz}

Example 10 - Modification of Rx to give broad specificity

This can be achieved as follows. A plasmid is assembled based on the Rx information disclosed herein, a pathogen-derived gene (selected in accordance with the desired specificity) and a reporter gene (GUS or GFP).

In controls the pathogen-derived gene may be the PVX CP. If the CP is from an avirulent isolate of PVX the Rx hair trigger will be activated leading to an HR. As a result of the HR there would be no GUS or GFP activity (the cells would be dead). In contrast if the CP is from a virulent strain there would be no HR and there would be a high level of GUS or GFP. However, a mutant Rx would recognize the virulent CP and there would be an HR and loss of GFP or GUS. To identify a mutant Rx that can recognize the virulent CP, Rx is mutagenised using PCR. The mutant forms of Rx will be inserted into the transient expression plasmid and expressed in plant cells and those capable of recognising the virulent CP will be diagnosed by the absence of GUS or GFP.

In the actual experiments, mutant forms of Rx are tested in an analogous manner for the ability to recognize proteins or molecules that are completely unrelated to the PVX CP.

Example 11 - Controlled activation of Rx-mediated broad specificity resistance

This may be achieved by any of the following methods:
(i) Expression of elicitor protein under the control of an inducible promoter in a plant carrying Rx. The activation of Rx mediated can be regulated by the extent to which the promoter is induced. The dex inducible promoter may be particularly suitable.

(ii) Expression of the elicitor coat protein from a replicating potato virus genome ('amplicon') (Angell and Baulcombe, 1997) in the background of Rx. Due to the Rx-mediated resistance the accumulation of the coat protein will be below the level required for elicitation of the HR but high enough to activate the resistance mechanism, as in infected plants.

(iii) Introduction of Rx into plants carrying a transgene that specifies the elicitor CP but in which the protein does not accumulate due to post transcriptional gene silencing (Baulcombe, 1996). In these plants the Rx-mediated resistance could be activated by any agent, for example a potyvirus, that is able to suppress post transcriptional gene silencing (Pruss et al., 1997). Thus following inoculation of these plants with a potyvirus the gene silencing would be suppressed, the Rx-mediated resistance would be activated and the continued accumulation and spread of the potyvirus would cease.

Examples 12 - the *Rx* locus in transgenic tobacco confers resistance not only to PVX but also to other Potex and Carlaviruses

By looking for homology between the coat protein of PVX and the coat protein of other viruses of the Potex- and Carlaviruses groups, we found sequence conservation within the coat protein (see Fig 8). To test whether *Rx* recognises a conserved elicitor domain we tested a series of viruses for their avirulence on transgenic *N. benthamiana* carrying the potato *Rx* gene. From this screen we found out that Narcissus mosaic virus (NMV), Nandina virus X (NVX), Viola mosaic virus (VMV), Cymbidium mosaic virus (CyMV), Poplar

mosaic virus (PopMV) and White clover mosaic virus (WClMV) were able to elicit *Rx*-mediated resistance. In these tests there were extreme resistance against NMV, NVX and WClMV and HR type resistance against VMV. Against CyMV and PopMV there was a weak elicitation of Rx-mediated resistance. These weak elicitation was not able to block the systemic mouvment of CyMV and PoMV. Their was no resistance against Foxtaim mosaic virus (FoMV). The implication of this finding is not only important at the fundamental level (identification of the conserved elicitor motif) but also at the applied level. Based on this study, it will be possible to create or select for a resistance gene that targets not only a single virus but also a class of viruses (example *Rx*).

GENERAL MATERIALS AND METHODS

1 Plant material

F1 seeds from the cross between potato cv Cara (*Rx, rx, rx, rx*) and cv Huinkel (*rx, rx, rx, rx*) were obtained from the Plant Breeding Institute, Cambridge. The progeny of this cross (370 individuals) were used initial experiments (Bendahmane *et al.*, 1997) to identify the chromosomal position of *Rx*, and to assign RFLP markers linked to *Rx*. The selfed (S1) progeny of cv Cara (1350 plants) were used as a mapping population to position *Rx* with respect to RFLP, AFLP and BAC-derived PCR markers. An additional 1438 plants of S1 cv Cara were used to ascertain whether BAC77 spans *Rx*. A dihaploid potato clone SH83-92-488 (Rouppe van der Voort *et al.*, 1997) derived from the cv Amaryl (*RxGpa2, rxgpa2*), cv Amaryl and selfed offspring of *S. tuberosum* spp. *andigena* breeding line CPC1673 (obtained from the Plant Breeding

Institute, Cambridge) were used for 'introgression mapping'.

2 Construction of the potato BAC library

High molecular weight DNA was prepared in agarose plugs from potato protoplasts essentially as described in Bendahmane *et al.* (1997). The agarose plugs were dialysed three times for 30 min against TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA), once at room temperature and twice at 4°C. The plugs were then equilibrated in *Hin*dIII buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, pH 7.9) twice on ice for 1 h. Half of each plug (~5 μg of DNA) was transferred to a test tube containing 360 μl of *Hin*dIII buffer and 10-15 units of *Hin*dIII restriction enzyme. The enzyme was allowed to diffuse into a plug at 4°C for 1 h and the digestion was carried out at 37°C for 30 min. The reaction was stopped by adding 1 ml of 0.5 M EDTA and plugs were immediately loaded into an 1% low melting point agarose gel and subjected to contour-clamped homogeneous electric fields (CHEF; Chu, 1989) electrophoresis in CHEF DR II system (Bio-Rad Laboratories, USA) in 0.5 X TBE buffer (45 mM Tris-borate pH 8.0, 1 mM EDTA) at 150 volts for 10 h at 4°C and constant pulse time of 5 sec or 8 sec. Compression zones containing the DNA fragments of ≥100 kb or ≥150 kb were excised from the gel and dialysed against 30 ml TE in a 15 cm Petri dish for 2 h at 4°C. Dialysed agarose slices then were transferred to an 1.5 ml test tube, melted at 65°C for 10 min and digested with 1 unit of GELASE (Epicentre Technologies, USA) per 100 μg of an agarose gel for 1 h at 45°C.

The size selected potato DNA (25-50 ng) was ligated to 25-50

ng of *Hin*dIII-digested and dephosphorylated pBeloBAC11 vector kindly provided by Dr H.Shizuya (University of Southern California, Los Angeles; Shizuya *et al.*, 1992) using 400 to 800 units of T4 DNA LIGASE (New England BioLabs, USA) at 16°C for 24 hours in a total volume of 50 μl. The ligation products were dialysed against 1 X TE using 0.025 μM MF-MILLIPORE MEMBRANE FILTER (Millipore, UK) at 4°C for 2 h and 30 min at room temperature using the "drop dialysis" method of Maruzyk and Sergeant (1980).

Transformation of *E. coli* DH10B cells was carried out by electroporation using a BRL CELL-PORATOR SYSTEM (Life Technologies Ltd, UK). To 20 μl of electro-competent cells, 0.5-3 μl of ligation mixture was added. After electroporation, *E. coli* cells were mixed with 1 ml SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated at 37°C for 1 h with gentle shaking (80 rpm). The cells were spread on Luria broth (LB) agar plates containing chloramphenicol (12.5 μg/ml), 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (Xgal) (40 μg/ml) and isopropyl-1-thio-ß-D-galactoside (IPTG) (0.12 mg/ml). Plates were incubated at 37°C for 24 hours.

DNA from the compression zones of 100 and 150 kb led to clones with an average insert size of 100 kb and a transformation efficiency of approximately 1000 and 150 white colonies per μl ligation, respectively. Approximately 92000 white colonies from these ligations were picked individually into 384 well microtiter plates (Genetix, UK) containing LB freezing buffer (36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 4.4 % V/V

glycerol, 12.5 μg/ml chloramphenicol in LB medium), grown overnight at 37°C and stored at -80°C. We have also prepared 100 bacterial pools containing ~500-1000 white colonies each (these pools also contained approximately 500-1500 blue bacterial colonies with an empty pBeloBAC11) by scraping the colonies from agar plates into the LB medium containing 18% glycerol and 12.5 μg/ml chloramphenicol using a sterile glass spreader. These pools were also stored at -80°C.

3 *Screening of the potato BAC library*

The potato BAC library was screened with IPM3, IPM4 and IPM5 CAPS markers corresponding to the AFLP markers PM3, PM4 and PM5 flanking the *Rx* locus (Bendahmane *et al.*, 1997). The IPM4F primer turned out to correspond to a portion of the C terminal part of the protein in a non-conserved part of the leucine rich repeat (bases 2522-2545 on Figure 3). The IMP4R primer corresponded to a region immediately downstream of the stop codon (base 2875 on Figure 3), extending into the intron immediately 5' of that codon. The protocol was as follows.

For the first part of the library of 92160 clones stored in 384 well microtiter plates the plasmid DNA was isolated using the standard alkaline lysis protocol (Heilig *et al.*, 1997) from pooled bacteria of each plate to produce 240 plate pools. Aliquots of these plate pools were combined to prepare 26 'superpools' each of which contains DNA from 9 plate pools, and one superpool containing DNA from 6 plate pools. To identify individual BAC clones carrying the CAPS markers the superpools and then the corresponding plate pools were screened. Once an individual plate had been

identified the clones corresponding to each of the 24 columns of the positive plate were grown for 3-4 h at 37°C in LB medium and PCR was carried out on 3 μl of bacteria. After identification of a positive column a colony PCR on each of the corresponding 16 colonies of this column was carried out leading to identification of a single positive BAC clone.

The second part of the library was stored as one hundred pools of approximately 1000 clones. The plasmid DNA was isolated from each pool of clones using standard alkaline lysis protocol and PCR was carried out to identify the positive pool. The bacteria corresponding to the positive pool were diluted, plated on LB agar plates and then colony hybridisation was carried out as described in Sambrook *et al.* (1989) using ^{32}P-labelled DNA probes corresponding to CAPS markers. PCR with the corresponding CAPS primers was used to distinguish hybridising colonies carrying the markers that had been previously mapped from the homologues located elsewhere in the genome.

4 Analysis of the BAC library

BACs containing potato DNA were isolated from 5 ml overnight cultures (LB medium supplemented with 12.5 mg/ml chloramphenicol) using the standard alkaline lysis miniprep protocol (Engebrecht *et al.*, 1997) and resuspended in 50 μl TE. Plasmid DNA (10 μl) was digested with *Not*I for 3 h at 37°C to free the genomic DNA from the pBeloBAC11 vector. The digested DNA was separated by CHEF electrophoresis in a 1% agarose gel in 0.5 X TBE at 4°C using a BIO-RAD CHEF DR II system (Bio-Rad Laboratories, USA) at 150 volts with a

constant pulse time of 14 sec for 16 h.

5 Isolation of BAC ends

Inverse polymerase chain reaction (IPCR; Ochman *et al.*, 1990) was used to isolate the right and left end sequences of insert DNAs. BAC DNA was isolated and digested separately with *Nla*III, *Hpa*II, *Mse*I, *Hin*P1I, *Pvu*II, *Hae*III (for isolation of a left end sequence) or with *Rsa*I, *Sac*I, *Eco*RI, *Hae*III, *Mae*II, *Mse*I, *Pvu*II, *Hin*P1I (for isolation of a right end sequence) for 4 h at 37°C and recircularised by self ligation for 2 h at 20°C. Ligations were carried out using 5-50 ng of digested DNA and 5-10 units of T4 DNA LIGASE (Boehringer Mannheim, Germany) in a total volume of 100 μl. PCR amplification of the recircularised DNA was carried out using 3 μl of self-ligated DNA as the template. AB1 (5'-CCTAAATAGCTTGGCGTAATCATG-3') and AB2 (5'-TGACACTATAGAATACTCAAGCTT-3') primers were used for PCR amplification of the left end sequence of insert DNA, AB3 (5'-CGACCTGCAGGCATGCAAGCTT-3') and AB4 (5'-ACTCTAGAGGATCCCCGGGTAC-3') primers were used for PCR amplification of the right end sequence of insert DNA. PCR conditions were as follows: 94°C for 15 sec, 60°C for 15 sec, 72°C for 90 sec - for 35 cycles. PCR products were digested simultaneously with *Hind*III and the restriction enzyme used in the preparation of IPCR DNA template. The released insert ends were gel purified and cloned into pGEM-3Z(f+) (Promega, USA). Sequences of the clones containing ~1-2 kb inserts were determined using a 377 or 373 DNA SEQUENCING SYSTEM (Applied Biosystems, UK), and these sequences were used to design PCR primers for amplification of the BAC end region in potato genomic DNA.

The sequences of primers and PCR conditions used in the amplification of each BAC end sequence are summarised in Table 1. The BAC end sequences were named according to the name of the BAC, followed by the letter L for the left end or by the letter R for the right end sequence. For example, the left and the right end sequences of BAC218 are 218L and 218R, respectively.

6 Viral cDNA clones and in vitro transcription

The PVX-TK and PVX-KR constructs are cDNAs of potato virus X (PVX) isolate CP4. PVX-TK has codons ACA (specifying T) and MA (specifying K) at positions 121 and 127 of the coat protein (CP) gene and is the same as wild-type CP4. The construct PVX-KR has MA (K) and AGG (R) at the coat protein codons 121 and 127, respectively. These constructs were described previously (Goulden et al., 1993). The tobacco mosaic virus (TMV) constructs were derived from the TMV vector (TMV/odontoglossum ringspot virus; construct TB2) described previously (Donson et al., 1991). The TMV-TK and TMV-KR were made by cloning PVX CP from PVX-TK and PVX-KR, respectively, in the Xhol site of TB2. Schematic structure of these constructs is described in Fig 6. The TMV-GFP was made by cloning the green fluorescent protein (GFP) open reading frame in the Xhol site of TB2.

7 Plasmid constructs for biolistic transient expression assay

PVX-TK/BAC77 and PVX-KR/BAC77 were made by digestion of the unique Srfl site in BAC77 and ligation with the cDNAs of the Rx-avirulent or the fix-virulent strain of PVX expressed

under the control of the cauliflower mosaic vinus (CaMV) 35S promoter. PVX in these constructs was defective in cell-to-cell movement and modified to express a 13-glucuronidase (GUS) reporter gene. The cell-to-cell movement defect was due to a sequence deletion between nucleotides 4588 and 4945 of the 25-kD protein open reading frame (Angell et al., 1 996). All the clones derived from PVX-TK/BAC77 and PVX-KR/BAC77 were designated PVX-TK/BAC* and PVX-KR/BAC* where the asterisks is used depending on the insert derivatives of BAC77 (see Figure 6).

8 Biolistic transient expression assay

Bacterial artificial chromosome (BAC) plasmid DNAs were isolated using an alkaline lysis method (Leonardo and Sedivy, 1990) and purified on cesium chloride gradients (Sambrook et al., 1989). One hundred micrograms of plasmid DNA was precipitated onto 10 mg of 0.95-lim diameter gold particles as described previously (McCabe et al., 1988). Leaf discs from 6-week-old plants, placed onto Murashige and Skoog medium (Imperial, UK) containing 3% (w/v) sucrose were bombarded with the gold particles coated with plasmid DNAs as described previously (McCabe et al., 1988). The leaf discs were incubated at room temperature in the dark for 48 hr and then stained for GUS activity as described previously (Angell and Baulcombe, 1997).

9 Agrobacterium tumefaciens-mediated transient expression (agroinfiltration)

The CP genes from the avirulent or the virulent strain of PVX were inserted between the 35S promoter and the

transcriptional terminator of CaMV and transferred into the binary vector pBin19 (Bevan, 1984) to create pBIN35S-TK and pBIN35S-KR, respectively. These constructs were transformed into Agrobacterium strain C58C1 carrying the virulence helper plasmid pCH32 (Hamilton et al., 1996). pCH32 expresses VirG and VirE and was used to enhance T-DNA transfer. Agrobacterium cells were inoculated into 5 mL L broth medium supplemented with 50 μg/mL kanamycin and 5 μg/mL tetracycline and grown at 28°C overnight. L broth medium (50 mL) supplemented with 50 μg/mL kanamycin, 5 μg/mL tetracycline, 10 mM MES pH 5.6, and 20 μM acetosyringone was then inoculated with the 5-mL overnight cultures and grown at 28°C for 1 day. Cells were precipitated and resuspended to a final concentration of 0.5 OD_{600} in a solution containing 10 mM MgCI2, 10 mM MES pH 5.6, and 10 μM acetosyringone. The cultures were incubated at room temperature for 2 hr before agroinfiltration. The agroinfiltration into *Nicotiana benthamiana* leaves was carried out as described previously (Scofield et al., 1996; Tang et al., 1996).

10 Protoplasts assay

Infectious viral RNA were electroporated into potato protoplasts and RNA gel blot analysis was used to measure the accumulation of viral RNA as described previously (Kohm et al., 1993). The probe used to detect PVX RNA was antisense RNA probe transcribed from the T7 promoter of pHB-RP plasmid linearized with Apal and was described previously (Kohm et al., 1993).

11 Graft Inoculation

Graft inoculations involved the grafting of healthy scions to infected stocks. First, seedlings of *N. benthamiana* were sap inoculated with PVX strains. Later, when the plants exhibited symptoms on the systemic leaves, the apex of a healthy plant to be tested was grafted onto the infected stock. A diagonal cut was made in the stem of the third internode to insert the infected scion. The 2 to 6 cm scions were given a V shape at the end that would be inserted into the cut on the stock plant to ensure good contact between vascular systems. The graft was secured with Parafilm M (Sigma) and the whole plant was covered with a thin transparent plastic bag to avoid dehydration of the scion. The bag was removed 7 days after grafting. The presence of virus in the stock and the scion was determined 4 weeks after grafting by using RNA gel blot analysis. All graft inoculation experiments were repeated at least twice.

12 Plant transformation with Rx

The Fspl-Pvull fragment of BAC9 (18,285 bp) was cloned into pSLJ7292 binary vector (Jones et al., 1992) digested with Ec113611 to create pSLJ9. This clone was introduced into Agrobacterium strain LBA4404. Transformation of *N. benthamiana* and *N. tabacum* were carried out by Agrobacteriummediated leaf disc transformation (Horsch et al., 1985). Transformation of potato cultivar Maris Bard was carried out as described previously (Gilbert et al., 1998). The transformation of *N. benthamiana* with the *N* disease resistance gene was carried out using the Agrobacterium strain AGL1 which carries the binary vector pTG34, and was kindly provided by B. Baker (University of Califomia, Berkeley, CA; Whitham et al., 1994).

13 DNA sequencing and analysis

A shotgun cloning strategy was used for sequencing BAC9. Three aliquots of 15 μg of CsCI purified DNA were sonicated for 30, 60 and 120 sec respectively by using Soniprep 150 (MSE, UK). DNA fragments in the range of 1-kb and 4-kb were gel purified, blunt ended by T4 DNA polymerase and cloned into pGEM3Zf+ (Promega) digested with Small Clones carrying potato insert DNA were selected by colony hybridization using insert DNA from BAC9 as probe. The sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer) and M13 universal forward and reverse primers. The sequence reactions were resolved on ABI377 automated sequencer (Applied Biosystems ABI, La Jolla, CA). Sequence contigs were assembled using UNIX versions of the Staden programs package (R. Staden, Medical Research Councel, Cambridge, UK). Homology searches were done using the BLAST software. The genomic DNA sequence has EMBL accession number AJ011801.

For the Rx homologues shown in Fig 7, the following primers were used for sequencing:

Rx specific primers used in the DNA sequencing:

K14: GCT ACC TCT ACG ATT TCA ACT TCC A
Rx5: GTA AAC TGA CAA GCG AGC TAG TT
Rx21: GAC ATA TGG ACT ACA GAA GCT TGG
Rx6: TAC CTG AAC TAG CAT ATT CAG CCA
K26: GTA GTA AAT TCC AAC TTT CG
K27: ACG AAA GTT GGA ATT TAC TAC
K2: ACC GAA CTT ACA TTT TCC CCA ATT C

Rx16: GCA TGA GAG TGT TGG CTT TGA GTT

Rx7: TTC AAA AAT CCC TCT ACA GGC CAT

Rx22: GTT CAC GGG TCA CAT CAT GCA TTC C

K29: GAG GGA AGC TCG AAA CAT G

Rx15: GTG ATC AAA ATT CAT GTG CAC AAT C

Rx8: AAT TGG CCA TGT ATT CAA ACC AAG

K20: TCA CAC TGG AAT TGT CTT TCA AGC

Rx23: TGA GCC TAC AGA GAA CAG ATT GGT

Rx24: ACC AAT CTG TTC TCT GTA GGC TCA

Rx9: TTT ACT CTT ACC TCC TCC GGA TGC T

K19: CTT CCA TGC CAC AGA GAA TTC TCC

K3: ACT CGA GGT CCT TAT ACT ATC ATG GA

K4: GAA CGA GTT ATT CTT AGA GAT TGC C

K17: GCT TGG CGG AAT TCA CAA CAG ATT

14 Rapid Amplification of cDNA Ends

The 5' and 3' ends of the RxcDNA were determined by rapid amplification of cDNA ends (RACE) using the MARATHON cDNA amplification kit (Clontech Laboratories, Palo Alto, CA). To obtain specific RACE products, two consecutive rounds of amplification (35 cycles each) were necessary. Conditions for the first round of polymerase chain reaction (PCR) were as recommended by the manufacturer. PCR samples of the first amplification round were diluted 1:10 with distilled water, and a 1-llL aliquot was used as a template for the second round of PCR (15 sec at 94°C, 15 sec at 65°C, and 1 min at 72°C). Two sets of oligonucleotides were used in combination with the adapter primers (AP1 and AP2) of the kit: K3 (5'-ACTCGAGGTCCTTATACTATCATGG-3') and K4 (5'-GAACGAGTTATTCTTAGATTGCCG-3') for the 3' end Of RX and oligonucleotides K14 (5'-GCTACCTCTACGATTTCAACTTCCA-3') and

K15 (5'GCAGGATTTCTCCAGAATAGCTCTCA-3') for the 5' end of Rx. RACE products were subcloned irto the pGEM-T plasmid (Promega) and sequences of 10 independent 3' and 5' end clones were determined asgiven above. Sequences of 5' and 3' RACE PCR products overlapped with the sequence of the PCR product corresponding to the central region of the *Rx* cDNA amplified using primers asK4 (5'-GAGAGCTATTCTGGAGAAATCCTGC-3') and asK15 (5'-GGCAATCTCTAAGAATAACTCGTTCA-3').

MATERIALS AND METHODS FOR CLONING RX HOMOLOGUES

15 Construction of pB1 binary vector

pB1 binary vector is a modified pBIN19 binary vector that carries a transcription cassette having an Rx promoter and Rx terminator. To construct the pB1 binary vector, first, the Rx promoter was PCR-amplified using the primers RxP4 (TCG GGG TAC CTC TAT TGA AGA ATT GAG ATC CAA G) and RxP2 (CTC AGT ATC TAG ATG AAC AAA TTG CC) and the PCR product was digested with XbaI. Second, the Rx terminator was PCR-amplified with primers RxT1 (CAG CTG TAA GCT CGT TGA TAT AGA GG) and RxT2 (GGT GTT CTA GAG ACT AGC CAG AGC TCT GAA AT) and the PCR product was digested with XbaI and KpnI. BAC77 DNA (Bendahmane et al 1999) was used as template for both PCR reactions. Third, the digested PCR products were ligated to a modified pBIN19 plasmid vector digested with KpnI and Ecl136 to create pB1. The modified pBIN19 plasmid is identical to the one published previously except the unique XbaI site was deleted.

16 Construction of a library of Rx homologues in pB1 binary vector for cloning Rx2

Rx homologues in *S. acaule* were PCR amplified using the primers Rx-1 (GGC AAT TTG TTC ATC TAG ATA CTG AGA GA) and Rxac-4 (TAT TTC AGA GCT CTG GCT AGT CCT CAG AAC ACC) that flank Rx gene. The PCR product was digested with XbaI and ligated to pB1 binary vector digested with XbaI and Ecl136. The library was made in *A. tumefaciens* strain C58C1 carrying the virulence helper plasmid pCH32 (Bendahmane et al 1999). Independent colonies were agroinfiltrated into tobacco leaves that express transgenically PVX coat protein (Spillane et al 1997) and screened for elicitation of hypersensitive response. We screened 200 colonies and 6 led to HR. Two positive clones pBAC15 and pBAC65 were sequenced and were found to carry the same insert DNA. There was only one amino acid polymorphic between pBAC15 and pBAC65, which may well have been introduced by the PCR.

17 Transgenic expression of the Rx2 candidate gene in Nicotiana species

The SmaI-Pvu II fragment of pBAC15 was cloned into pSLJ7292 binary vector (Jones et al., 1992) digested with Ecl 136II to create pSLJAC15. This clone was introduced into *Agrobacterium tumefaciens* strain LBA4404. Transformation of *N. benthamiana* and *N. tabacum* were carried out by *A. tumefaciens*-mediated leaf disc transformation (Horsch et al., 1985). The transgenic plants were tested for resistance to virulent and avirulent strain of PVX by mechanical inoculation. Four independent transgenic lines of N. tabacum and two independent transgenic lines of N. benthamiana carrying the SmaI-Pvu II fragment of pBAC15 regenerated were resistant to *Rx*-avirulent PVX-TK and susceptible to virulent PVX-KR. This resistance was manifested as the absence of

PVX-TK accumulation in the inoculated and systemic leaves.

MATERIALS AND METHODS FOR OVEREXPRESSING RX

18 Construction of pBIN35-Rx binary vector for Rx overexpression assay

The Construction of pBIN35-Rx binary vector was carried out using pBIN61 binary vector (see *15* above). Rx cDNA was PCR amplified with the primers Rx1 (GGC AAT TTG TTC ATC TAG ATA CTG AGA GA) and Rxac4 (TAT TTC AGA GCT CTG GCT AGT CCT CAG AAC ACC). The PCR product was digested with XbaI and ligated with pBIN61 digested with XbaI and SmaI to create pBIN35-Rx.

19 Construction of pB1-Rx binary vector

The Construction of pB1-Rx binary vector was carried out using pB1 binary vector. pB1 binary vector is a modified pBIN19 binary vector that carry a tanscriprion cassette constituted of Rx promoter and Rx terminator. To Construct pB1 binary vector, first, the Rx promoter was PCR-amplified using the primers RxP4 (TCG GGG TAC CTC TAT TGA AGA ATT GAG ATC CAA G) and RxP2 (CTC AGT ATC TAG ATG AAC AAA TTG CC) and the PCR product was digested with XbaI. Second, the Rx terminator was PCR-amplified with primers RxT1 (CAG CTG TAA GCT CGT TGA TAT AGA GG) and RxT2 (GGT GTT CTA GAG ACT AGC CAG AGC TCT GAA AT) and the PCR product was digested with XbaI and KpnI. BAC77 DNA (Bendahmane et al 1999) was used as template for both PCR. Third, the digested PCR products were ligated to a modified pBIN19 plasmid vector digested with KpnI and Ecl136 to create pB1. The modified pBIN19 plasmid is identical to the one published previuosely except the

unique XbaI site was deleted.

To construct pB1-Rx, Rx cDNA was PCR amplified with the primers Rx1 (GGC AAT TTG TTC ATC TAG ATA CTG AGA GA) and Rxac4 (TAT TTC AGA GCT CTG GCT AGT CCT CAG AAC ACC). The PCR product was digested with XbaI and SacI and ligated with pB1 digested with XbaI and SacI to create pB1-Rx.

20 Agrobacterium-mediated transient expression (agroinfiltration)

The constructs pBIN35-Rx and pB1-Rx were transformed into *A. tumefaciens* strain C58C1 carrying the virulence helper plasmid pCH32 (Hamilton et al., 1996). pCH32 expresses VirG and VirE and was used to enhance T-DNA transfer. Agrobacterium cells were inoculated into 5 ml L-broth medium supplemented with 50 μg/ml kanamycin and 5 μg/ml tetracycline and grown at 28°C overnight. The Cells were then precipitated and resuspended to a final concentration of 0.5 OD_{600} in a solution containing 10 mM $MgCl_2$, 10 mM MES pH 5.6 and 150 μM acetosyringone. The cultures were incubated at room temperature for 2 hr before agroinfiltration into *N. tabacum* leaves.

21 Challenge of transgenic plants with Potex- and Carlaviruses

All the viruses used in this experiment were maintaned in wild type *N. benthamiana*. To carry out the resistance test, for each virus, one leaf of an infected *N. benthamiana* plant was ground in a pestle and mortar in presence of 1 ml of 10 mM phosphate buffer and sand. The solution was centrifuged for 2 min at 2000 g and the supernatant was used as inoculum

to infect Rx transgenic *N. benthamiana*. As a control we also inoculated wild type *N. benthamiana*. The infected plants were scored for resistance 3 weaks post-inoculation. The plant were considered resistant if there were no symptoms and no virus accumulation in the inoculated and the systemic leaves of infected Rx transgenic plants and symptoms and systemic movement of the virus in the control plant (infected wild type *N. benthamiana*).

Annex I - Rx DNA sequence - nt 1-5820 - rows of 80

ACTACTTACACTTATACATGGTATAAGAATTTTGCACAATTACTTACATATATACAATAT
TATCAATTAAACAATATACA
AATCGTATAACTTATATATACAGTAAAATTACAACAACAACAACAAAAATTATCAAATTA
AAGCACACCGTTGTTGTCGA
ATCATATACACTTCATATATAAAAATTGTGTCATTCAATTTTTCGAACAAAAAATTAGAA
TTGAATTGGTAATAAAAAAT
TTATCTAATCTTGTATAAACAAAATTAAATTATTGCAAACCATTAGAATGAAAAAAACAA
AAATAATCCGTTTTCCAAAA
TTTCAATTATATACTATACAAATCAATTGTATACTTTCTTGCTGTTCAAAACATGAAGTT
TCCTTGAAAGAAACGCTTAC
CTAGCGTTGAATATACAAGAATATTGATTAATCTTATGCTTCAGTCGTTTGAGGAACCCA
GTTGTTATGGTGTTTCTATT
GCTATAGAACTCCTTTTTGGAAAAATATTTGATTTTGGACGATTAGCTTGAACATTGGGA
CTATATAAATTTTTTATTAC
CGTATTTAGCACTCATGTATCCATTTATTAAAAAAAAAATGTATAAATTATATTTTTAAAA
GAAAATATACAAAATTAATG
CTTCATAGCAAACTAAACTATGCCCATTGAATGTAATTACTAAACTATACCTATAGAGCG
TTATTTCATTAAATACGTTT
ATCATATATGAAATTTTCCCTTAAGAGATCCTACACCTTATATATAGCTTCTCAAATGTG
GAAATTCAATCTCACACCCA
ACAATCTTTCCCTCAGACTAAGTTTCATGGCCCAATATCACAATGATCCACGAGTCAATT
CATGAGATTCACTATGTGCA
TCACCCACATCGTCTAAGTATTTTATGGCAATCAAGCCCTACAACTAGCTTCTTCTTTAT
ATATATATGTGTGTGTATAT
GTGCGCGCGCGCGCATCTCTAATTAATCTCGTAAAGGGATTAAGGGGCCAATTTCAAAGA
ATTAGGCGATTTTCTTAGTT
TTTCGTGTGTGTTAACCCATAAATATTTTGGTGATATGGTTTTCGGACGATTTCTTTTGT
GCAACTTATATGGAACCCTT
CGTAGGGAGTTAGTCTCACACTTTTTAGAGTCCATTTTGGGCACTCAGGGGCTAATTTAT
AGGAAATAGGTGATCTTCTC

AGTCCGTCTGTATTAGCCCATGAATATTTTGGTGATATGTCTTCCGAATAATTTCTTTGT
AAAATCTTTACGGGACCCTC
CATAGGGAGTTAATGGAGCAGTACGTATAGTCTCACAATTTTAGAGTTCATTTTGGGCAT
TTAGGGGCCAATTTACAGGA
ATTAGGTGACTTTCTCAGTGTTTTGTGTGTGTTAGCCCATTAATATTTGGTGATATGACT
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CACATTTACGAAACCCTCTGTAGGGAGTCGGGGGAGCAGTACGTACAATCTCACAATTTT
AGAGTCCATTTTAGACATTT
AGGGGCCAATTTAAAGAAATTGGACAATTTTCTCAGTTTTTCGTGTCTGTTAGCCATTAA
TATATTGGTGAATATGACCT
ACGGATGATTTCTAATAGAAATCTTTACGAAACCTTCAATAGGGAGTTGGGGGAGCAATA
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TTTCCATGTGTTAGCCCATA
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TGCTTGGTTAATTGAATCGA
ACTAGGAATAGAGAAATTCCTACTTGGATCTTAATATTTCTCTCTCTTTGATTTGGAAAA
TTCTACGAAGTTGCTTTCAA
TGGAATTAAAATCATAAATCTATTGTATGTAAGAAACATACTTATATTCATGAATAGATA
TGTGTAGGGTCTAATAATGA
ATTATCACAATTTTTTCTACTTTTTCCTGTCAGAGTCCTGCTTTTTCTTTTTCTTTTTCT
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AACAATCTTGGAAGTTGAAATCGTAGAGGTAGCATACACAACAGAAGATATGGTTGACTC
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TAGCACAGAATTTGGAGGAAAGAAGCAGGGCTATGTGGGAGATTTTTTTCGTCCTGGAAC
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GAGGAGGAAGGGAACTAGAAGTTGTCTCAATCGTAGGGATGGGAGGCATCGGGAAAACAA
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TATTGTGTGAGAAATGTACT
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GGAAGCAGAATACTCCTGACTACTCGGAATGTGGAAGTGGCTGAATATGCTAGTTCAGGT
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CCTCATGAATTTTGACGAAAGTTGGAATTTACTACACAAAAAGATCTTTGAAAAAGAAGG
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TTGCTGGACTTCTCTCCAAA
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CATGAGAGTGTTGGCTTTGAGTTACCATCACTTGCCTTCTCACCTAAAACCGTGTTTTCT
GTATTTTGCAATTTTCACAG
AGGATGAACAGATTTCTGTAAATGAACTTGTTGAGTTATGGCCTGTAGAGGGATTTTTGA
ATGAAGAAGAGGGAAAAAGC
ATAGAAGAGGTGGCAACAACATGTATAAACGAACTTATAGATAGAAGCTTAATTTTCATC
CACAATTTTAGTTTTCGTGG
AACAATAGAAAGTTGTGGAATGCATGATGTGACCCGTGAACTCTGTTTGAGGGAAGCTCG
AAACATGAATTTTGTGAATG
TTATCAGAGGAAAGAGTGATCAAAATTCATGTGCACAATCCATGCAGCGTTCCTTTAAGA
GTCGAAGTCGGATCAGAATC

CATAAGGTGGAAGAATTGGCTTGGTGTCGTAACAGTGAGGCTCATTCTATTATCATGTTG
GGTGGATTCGAATGCGTCAC
ACTGGAATTGTCTTTCAAGCTAGTAAGAGTACTAGATCTTGGTTTGAATACATGGCCAAT
TTTTCCCAGTGGAGTACTTT
CTCTAATTCATTTGAGATACCTATCTTTGCGTTTTAATCCTTGCTTACAGCAGTATCAAG
GATCGAAAGAAGCTGTTCCC
TCATCAATAATAGACATTCCTCTATCGATATCAAGCCTATGCTATCTGCAAACTTTTAAA
CTTAACCTTCCATTTCCCAG
TTATTATCCTTTCATATTACCATCGGAAATTTTGACGATGCCACAATTGAGGACGCTGTG
TATGGGCTGGAATTACTTGC
GGAGTCATGAGCCTACAGAGAACAGATTGGTTTTGAAAAATTTGCAATGCCTCAATCAAT
TGAACCCTCGGTATTGTACA
GGGTCTTTTTTTAGACTATTTCCCAATTTAAAGAAGTTGCAAGTATTTGGCGTCCCAGAA
GACTTTCGCAATAGCCAGGA
CCTGTATGATTTTCGCTACTTATATCAGCTCGAAGAATTGACATTTCGTTTATATTATCC
ATATGCTGCTTGCTTTCTAA
AAAACACTGCACCTTCAGGTTCTACGCAAGATCCTCTGAGGTTTCAGACGGAAATATTGC
ACAAAGAGATTGATTTCGGG
GGAACTGCACCTCCAACTTTACTCTTACCTCCTCCGGATGCTTTTCCACAAAACCTTAAG
AGTTTAACTTTTAGGGGAGA
ATTCTCTGTGGCATGGAAGGATTTGAGCATTGTTGGTAAATTACCCAAACTCGAGGTCCT
TATACTATCATGGAATGCCT
TCATAGGCAAGGAGTGGGAAGTAGTTGAGGAAGGGTTTCCTCACTTGAAGTTCTTGTTTC
TGGATGATGTATACATTCGA
TACTGGAGAGCTAGTAGTGATCACTTTCCGTACCTTGAACGAGTTATTCTTAGAGATTGC
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TCGAGATTTTGCAGATATAACCACACTAGCTCTTATTGATATAGATTACTGTCAACAATC
TGTTGTGAATTCCGCCAAGC
AAATTCAACAGGACATTCAAGACAACTATGGAAGCTCTATCGAGGTCCATACTCGTCATC
TTTTGTAAGACATCTTCTTC
CTTGCTTTACAACAATAATTAACTCATCATCATAGTAAACTCGATAATAATCTGGATAAT
AGCCTTAGTAAGTCAAATTG

CACCAATTCAACAAAAGTTCTTGATGCTGTCATTGTGTTTGATTTGAATCCTTCCAATAT
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CATGTTCATTCTTGATTTTGGGAAGTGTAACATTTCATTTTTCATCTTTTGTGGCTAGCA
TTCCCAAGAGTGTGACAACA
GTTGAAGATGATGATGATAGTGTGACAACAGATGAAGATGATGATGATGATGACTTTGAG
AAAGAAGTTGCTTCTTGCCG
CAATAATGTGTAAGTTCTTATACCTGCATGCTCATTCTTGCTATAATGTTCTCTTGTTCC
TTAATTATGGGACATCTGAC
ATATTATTTTCCATGTTTTGCGTCTTTTATTTTTCTGCAGCGAGTAGTTAAGGTGTTCTG
AGGACTAGCCAGTTCTCTGA
AATAAATGTCAAATCAGAAGCCAAATGTGTGAGTGTTTGTTTTGTTCGTTTTCATTTTTT
CTGCATAAGGTGGCAGGATG
ATTGCAAATGGCTTGTAATTTAATTGTATATGATCTTTCGTATAGCCATTTGTCAGTGGT
TCTTAAGATACTCCAAATTT
TATGCACATACATACATACTGTACAGGCCAGAACAGACTCCAGTAACGTGTGTTTCCTTT
CTTGGGAGTCCTCAATCTAC
CTCGCAAAGGCTAAATCCAGTGGCACCAGCTTTATTACTAAAACATTCACACGGGAACAG
TTGAGAAAAACTAGGCCTCC
ATACCAAACACACCCTTAAACTTGAGCTGGTTGATAGAGACTAGAGAGTAGAGAGCACTA

Annex II - Rx cDNA sequence - nt 1-3066

TGGCAATTTGTTCATTTTCATACTGAGAGATATTTCTATTTT
TTGGATATATGGCTTATGCTGCTGTTACTTCCCTTATGAGAACCATACATCAATCAATGG
AACTTACTGGATGTGATTTG
CAACCGTTTTATGAAAAGCTCAAATCTTTGAGAGCTATTCTGGAGAAATCCTGCAATATA
ATGGGCGATCATGAGGGGTT
AACAATCTTGGAAGTTGAAATCGTAGAGGTAGCATACACAACAGAAGATATGGTTGACTC
GGAATCAAGAAATGTTTTTT
TAGCACAGAATTTGGAGGAAAGAAGCAGGGCTATGTGGGAGATTTTTTTCGTCCTGGAAC
AAGCACTAGAATGCATTGAT

TCCACCGTGAAACAGTGGATGGCAACATCGGACAGCATGAAAGATCTAAAACCACAAACT
AGCTCGCTTGTCAGTTTACC
TGAACATGATGTTGAGCAGCCCGAGAATATAATGGTTGGCCGTGAAAATGAATTTGAGAT
GATGCTGGATCAACTTGCTA
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AGTGATCCGTGCATTATGTCTCGATTTGATATTCGTGCAAAAGCAACTGTTTCACAAGAG
TATTGTGTGAGAAATGTACT
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GATACTTGGTAGTCATTGATGACATATGGACTACAGAAGCTTGGGATGATATAAAACTAT
GTTTCCCAGACTGTTATAAT
GGAAGCAGAATACTCCTGACTACTCGGAATGTGGAAGTGGCTGAATATGCTAGTTCAGGT
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CCTCATGAATTTTGACGAAAGTTGGAATTTACTACACAAAAAGATCTTTGAAAAAGAAGG
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AAAATATTGGGAAACAAATTGCATTAAAATGTGGAGGATTACCTCTAGCAATTACTGTGA
TTGCTGGACTTCTCTCCAAA
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CATGAGAGTGTTGGCTTTGAGTTACCATCACTTGCCTTCTCACCTAAAACCGTGTTTTCT
GTATTTTGCAATTTTCACAG
AGGATGAACAGATTTCTGTAAATGAACTTGTTGAGTTATGGCCTGTAGAGGGATTTTTGA
ATGAAGAAGAGGGAAAAAGC
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CACAATTTTAGTTTTCGTGG
AACAATAGAAAGTTGTGGAATGCATGATGTGACCCGTGAACTCTGTTTGAGGGAAGCTCG
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TTATCAGAGGAAAGAGTGATCAAAATTCATGTGCACAATCCATGCAGCGTTCCTTTAAGA
GTCGAAGTCGGATCAGAATC
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GGTGGATTCGAATGCGTCAC

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CTTAACCTTCCATTTCCCAG
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TATGGGCTGGAATTACTTGC
GGAGTCATGAGCCTACAGAGAACAGATTGGTTTTGAAAAATTTGCAATGCCTCAATCAAT
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GACTTTCGCAATAGCCAGGA
CCTGTATGATTTTCGCTACTTATATCAGCTCGAAGAATTGACATTTCGTTTATATTATCC
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ACAAAGAGATTGATTTCGGG
GGAACTGCACCTCCAACTTTACTCTTACCTCCTCCGGATGCTTTTCCACAAAACCTTAAG
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CTGCATAAGGTGGCAGGATG
ATTGCAAATGGCTTGTAATTTAATTGTATATGATCTTTCGTATAGCCATTTGTCAGTGGT
TCTTAAGATACTCCAAATTT
TATGCACAT

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Claims

1. An isolated nucleic acid encoding a polypeptide which is capable of conferring extreme resistance against a pathogen in a plant in which said polypeptide is expressed.

2. A nucleic acid as claimed in claim 1 wherein the pathogen is a plant virus.

3. A nucleic acid as claimed in claim 1 or claim 2 comprising an Rx polynucleotide sequence from *Solanum tuberosum*.

4. A nucleic acid as claimed in claim 3comprising a nucleotide sequence identical to Seq ID No 1 or Seq ID No 3 or being degeneratively equivalent thereto.

5. A nucleic acid as claimed in claim 3 or claim 4 wherein the nucleotide sequence encodes the Rx polypeptide of Seq ID No 2.

6. An isolated nucleic acid encoding a homologous variant of a nucleotide sequence of any one of claims 3 to 5 having about 50% or more sequence identity therewith and encoding a polypeptide capable of conferring resistance against a pathogen in a plant.

7. A nucleic acid as claimed in claim 6 wherein the resistance conferred is extreme resistance.

8. A nucleic acid as claimed in claim 6 or claim 7 wherein the variant is selected from, or degeneratively equivalent

to: an Rx homologue; an Rx allelic variant; an Rx orthologue; or an Rx paralogue, which is in each case obtainable from a plant species.

9. A nucleic acid as claimed in claim 8 wherein the variant is an *Rx*-linked homologue from a *Solanum* species.

10. A nucleic acid as claimed in claim 8 or claim 9 wherein the variant is selected from: 111h1; 221h2; Ac15; Ac64; K39.hom as shown in Fig 7.

11. A nucleic acid as claimed in claim 6 or claim 7 wherein the variant is a homologous resistance gene obtainable from a plant other than *Solanum* spp.

12. A nucleic acid as claimed in claim 6 or claim 7 wherein the variant is a derivative of a nucleotide sequence of any one of claims 3 to 5 obtainable therefrom by way of nucleotide addition, insertion, or substitution.

13. A nucleic acid as claimed in claim 12 which encodes a resistance polypeptide which is modified with respect to its activated by an elicitor

14. A nucleic acid which is complementary to the nucleic acid of any one of claims 1 to 13.

15. An isolated nucleic acid molecule for use as a probe or primer, said molecule having a nucleotide sequence at least 14, 18, 21, or 24 nucleotides in length, which sequence is present in, or complementary to, a nucleic acid as claimed in any one of claims 3 to 5, or claim 10.

16. A nucleic acid as claimed in claim 15 which is a primer consisting of 30 or fewer nucleotides.

17. A nucleic acid as claimed in claim 15 or claim 16 which does not encode all or part of the following motifs of the Rx polypeptide of Seq ID No 2 in Fig 1: a leucine zipper; a nucleotide binding site (NBS); a leucine rich repeat (LRR).

18. A nucleic acid as claimed in any one of claims 15 to 17 which encodes all or part of the any of the following motifs of the Rx of Seq ID No 2 in Fig 1: the amide-rich region; the short basic region; the acidic tail region; an Rx gene signature region.

19. A nucleic acid as claimed in any one of claims 15 to 18 which is selected from the following primers:

K14: GCT ACC TCT ACG ATT TCA ACT TCC A
Rx5: GTA AAC TGA CAA GCG AGC TAG TT
Rx21: GAC ATA TGG ACT ACA GAA GCT TGG
Rx6: TAC CTG AAC TAG CAT ATT CAG CCA
K26: GTA GTA AAT TCC AAC TTT CG
K27: ACG AAA GTT GGA ATT TAC TAC
K2: ACC GAA CTT ACA TTT TCC CCA ATT C
Rx16: GCA TGA GAG TGT TGG CTT TGA GTT
Rx7: TTC AAA AAT CCC TCT ACA GGC CAT
Rx22: GTT CAC GGG TCA CAT CAT GCA TTC C
K29: GAG GGA AGC TCG AAA CAT G
Rx15: GTG ATC AAA ATT CAT GTG CAC AAT C
Rx8: AAT TGG CCA TGT ATT CAA ACC AAG
K20: TCA CAC TGG AAT TGT CTT TCA AGC
Rx23: TGA GCC TAC AGA GAA CAG ATT GGT

Rx24: ACC AAT CTG TTC TCT GTA GGC TCA
Rx9: TTT ACT CTT ACC TCC TCC GGA TGC T
K19: CTT CCA TGC CAC AGA GAA TTC TCC
K3: ACT CGA GGT CCT TAT ACT ATC ATG GA
K4: GAA CGA GTT ATT CTT AGA GAT TGC C
K17: GCT TGG CGG AAT TCA CAA CAG ATT

Rx-1: GGC AAT TTG TTC ATC TAG ATA CTG AGA GA
Rxac-4: TAT TTC AGA GCT CTG GCT AGT CCT CAG AAC ACC

20. A method for selecting a plant comprising a pathogen resistance gene from a plant, which method employs a probe or primer as claimed in any one of claims 15 to 19.

21. A method for identifying or cloning a pathogen resistance gene from a plant, which method employs a probe or primer as claimed in any one of claims 15 to 19.

22. A method as claimed in claim 21 for isolating a homologue as claimed in any one of claims 8 to 11, comprising the steps of:
i) producing a plant population in which a resistance trait is segregating.
ii) amplifying DNA from individual members of the population with one or more primers as claimed in any one of claims 15 to 19.
iii) testing the PCR products for sequence polymorphism that co-segregates with the resistance trait.

23. A method for producing a nucleic acid encoding an Rx derivative comprising the step of modifying a nucleic acid as claimed in any one of claims 1 to 11.

24. A recombinant vector comprising a nucleic acid of any one of claims 1 to 13, which nucleic acid encodes a resistance polypeptide.

25. A vector as claimed in claim 24 which is capable of replicating in a suitable host.

26. A vector as claimed in claim 24 or claim 25 wherein the nucleic acid is operably linked to a promoter or other regulatory element for transcription in a host cell.

27. A vector as claimed in claim 26 further comprising any one or more of the following: a terminator sequence; a polyadenylation sequence; an enhancer sequence; a marker gene.

28. A vector as claimed in claim 26 or claim 27 wherein the promoter is an inducible promoter.

29. A vector as claimed in any one of claims 25 to 28 which is a plant vector.

30. A vector as claimed in any one of claims 24 to 29 further comprising a sequence encoding an elicitor for the encoded resistance polypeptide.

31. A vector as claimed in claim 30, wherein the elicitor sequence encodes all or part of the coat protein of a virus selected from: Potato Virus X (PVX); Narcissus mosaic virus (NMV); Nandina virus X (NVX); Viola mosaic virus (VMV); Cymbidium mosaic virus (CyMV); Poplar mosaic virus (PopMV) and White clover mosaic virus (WClMV).

32. A vector as claimed in claim 30 or claim 31 wherein transcription or translation of the elicitor is suppressed by the activation of the resistance polypeptide.

33. A vector as claimed in claim 30 or claim 31 wherein transcription or translation of the elicitor is activated in the presence of the pathogen against which the resistance polypeptide confers resistance.

34. A vector as claimed in claim 30 or claim 31 wherein transcription or translation of the elicitor or the resistance polypeptide is activated in a variegated manner.

35. A method comprising the step of introducing a vector as claimed in any one of claims 25 to 34 into a cell.

36. A method for transforming a plant cell, comprising a method as claimed in claim 35, and further comprising the step of causing or allowing recombination between the vector and the plant cell genome to introduce the nucleic acid into the genome.

37. A host cell comprising a vector as claimed in any one of claims 24 to 34.

38. A host cell transformed with a vector as claimed in any one of claims 24 to 34.

39. A host cell as claimed in claim 37 or claim 38 which is a plant cell.

40. A host cell as claimed in claim 39 which is in a plant.

41. A method for producing a transgenic plant comprising a method as claimed in claim 36 and further comprising the step of regenerating a plant from the transformed cell.

42. A plant obtainable by the method of claim 41, which plant comprises the cell of claim 39 or claim 40.

43. A plant which is the progeny of a plant as claimed in claim 42, which plant comprises the cell of claim 39 or claim 40.

44. A part or propagule of the plant of claim 42 or claim 43, which part or propagule comprises the cell of claim 39 or claim 40.

45. A resistance polypeptide encoded by the nucleic acid of any one of claims 1 to 13.

46. A method of producing a polypeptide comprising the step of causing or allowing the expression from a nucleic acid of any one of claims 1 to 13 in a suitable host cell.

47. A composition comprising the polypeptide of claim 46.

48. An antibody or fragment thereof, or a polypeptide comprising the antigen-binding domain of the antibody, capable of specifically binding the polypeptide of claim 46.

49. A method for influencing or affecting a resistance trait in a plant, the method comprising use of any one or more of the following: all or part of the nucleic acid of

any one of claims 1 to 14; the polypeptide of claim 46; the antibody or fragment or polypeptide comprising the antigen-binding site thereof of claim 48.

50. A method as claimed in claim 49 comprising the step of causing or allowing expression of a nucleic acid according to any one of claims 1 to 13, which nucleic acid encodes a resistance polypeptide, within a cell of that plant.

51. A method as claimed in claim 50 comprising constitutively over-expressing the resistance polypeptide in the plant.

52. A method as claimed in claim 50 or claim 51 wherein the resistance polypeptide is triggered by contact with an appropriate elicitor or inducer.

53. A method as claimed in claim 52 wherein the expression of the elicitor is under the control of an inducible promoter whereby the activation of the resistance polypeptide can be regulated by the extent to which the promoter is induced.

54. A method as claimed in claim 52 wherein the elicitor post transcriptionally gene silenced whereby the activation of the resistance polypeptide can be regulated by suppressing the post transcriptional gene silencing.

55. A method for establishing gene for gene compatibility between an elicitor and a resistance polypeptide encoded by a nucleic acid as claimed in any one of claims 1 to 13, which method includes the steps of:

(a) causing or permitting the co-expression in cell of the resistance polypeptide with the elicitor,
(b) observing said cell for an HR,
(c) correlating the result of the observation made in (b) with the compatibility of the elicitor and the resistance polypeptide.

56. An isolated nucleic acid comprising a nucleotide sequence encoding the promoter region of the gene encoding the resistance polypeptide of claim 1 or claim 10.

MAYAAVTSLMRTIHQSMELTGCDLQPFYEKLKSLRAI
LEKSCNIMGDHEGLTILEVEIVEVAYTTEDMVDSESR
NVFLAQNLEERSRAMWEIFFVLEQALECIDSTVKQWM
ATSDSMKDLKPQTSSLVSLPEHDVEQPENIMVGRENE

FEMMLDQLARGGRELEVVSIVGMGGIGKTTLAT — kinase motiff 1a

KLYSDPCIMSRFDIRAKATVSQEYCVRNVLLGLLSLT
SDEPDDQLADRLQKHLKGRRYLVVIDDIWTTEAW — kinase motiff 2

DDIKLCFPDCYNGSRILLTTRNVEVAEYASSGKP — kinase motiff 3a

PHHMRLMNFDESWNLLHKKIFEKEGSYSPEFENIGKQ
IALKCGGLPLAITVIAGLLSKMGQRLDEWQRIG — R gene signature 1

ENVSSVVSTDPEAQCMRVLALSYHHLPSH
LKPCFLYFAIFTEDEQISVNELVELWPVEGFLNE — R gene signature 2

EEGKSIEEVATTCINELIDRSLIFIHNFSFRGTIESCG
MHDVTRELCLREARN — R gene signature 3

MNFVNVIRG KSDQNSCAQS MQRSFKSRSR
IR IHKVEELAWCRNSEAHS
IIMLGGFECVIL
ELSFKLVRVLDLGLN TW PIFPSG
VLSLIHLRYLSLRFNPCLQQYQGSKEAVPSSIIDIPLS
ISSLCYLQTFKLNL PFPSYYPFILPSE
ILTMPQLRTLCMGWN YLRSHEPTENRLV
LKNLQCLNQLNPRYCTGSF
FRLFPNLKKLQVFGVPEDFRNSQDLYD
FRYLYQLEELIFRLYYPYAACFLKNTAPSGSTQDPLRF
QTEILH KEIDFGGTAPPTLLLPPP
DAFPQNLKSLIFRGEFSVAWKDLSI
VGKLPKLEVLILSWNAFIGKEWEVV
EEGFPHLKFLFLDD VYIRYWRAS
SDHFPYLERVILRDCRNLD SIPRD
FADITILALIDIDYC

{axx axx L xx Lx Lxx N xxa xxx aPxx} LRR consensus
C
T

QQSVVNSAKQIQQDIQDNYGSSIEV — Amide(QN)-rich

HTRHLFIPK — Basic (+)

SVTTVEDDDDSVTTDEDDDDDDFEKEVASCRNNVE — Acidic (-)

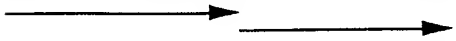

Figure 1

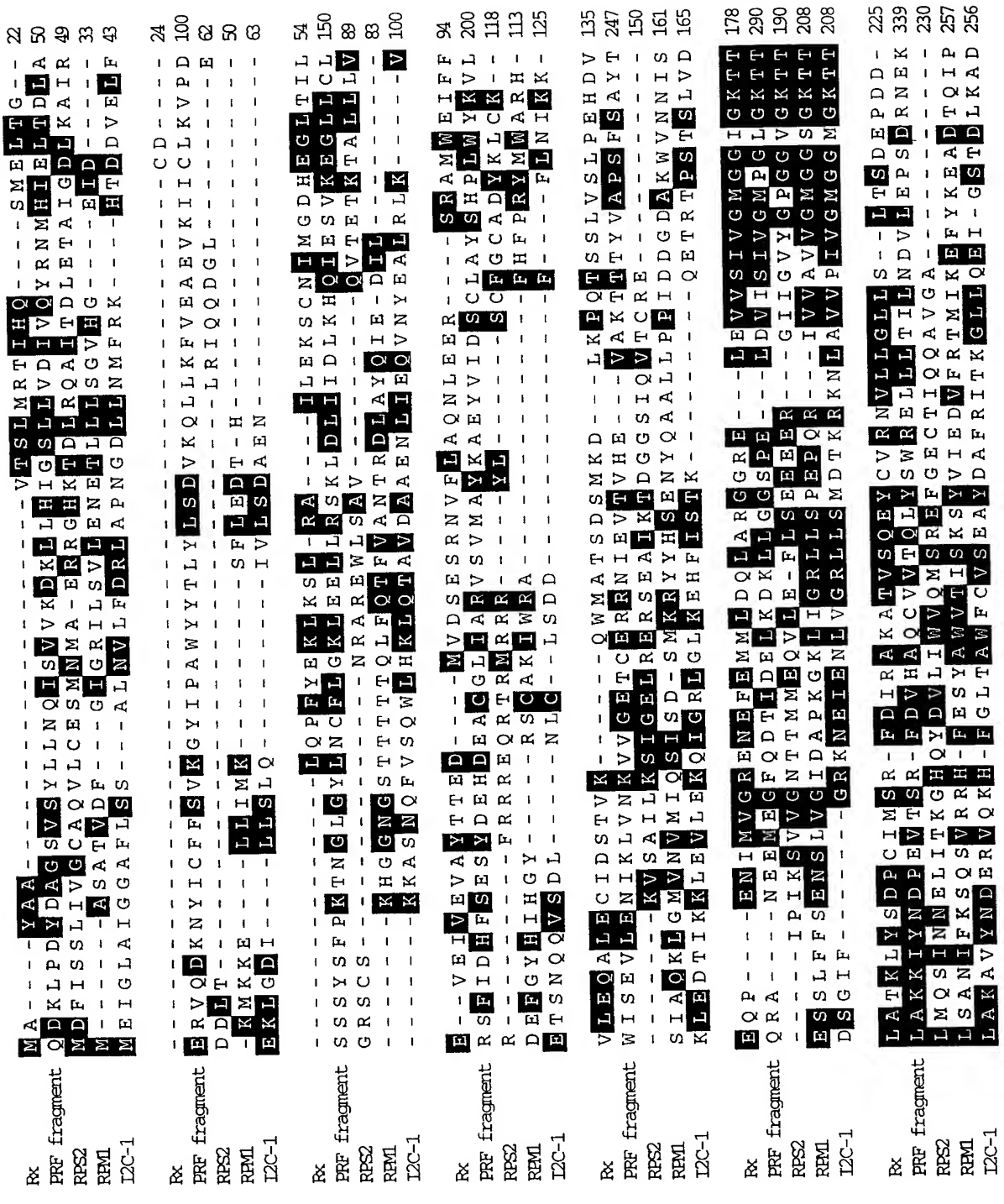

Figure 2

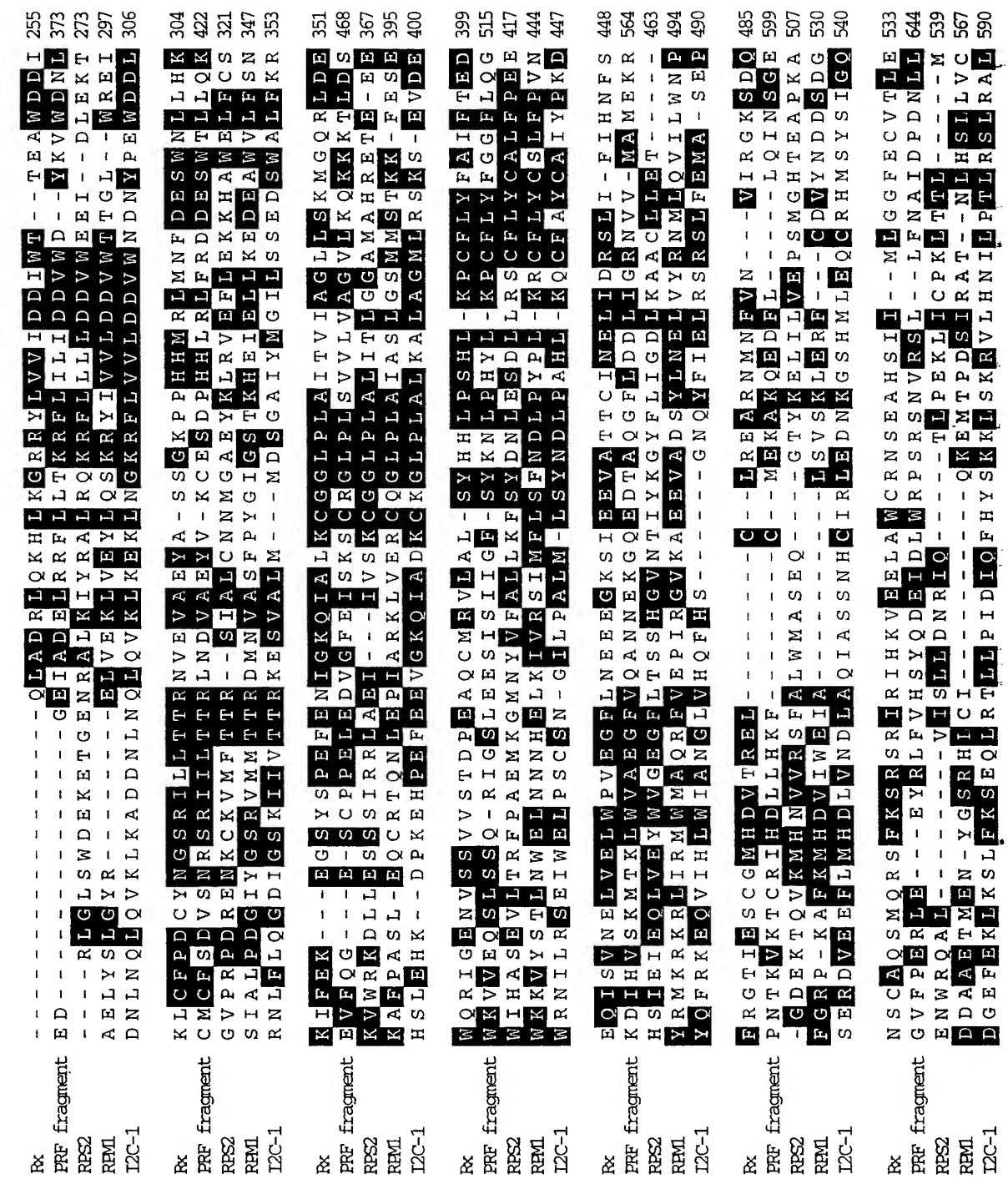

Figure 2 ...cont

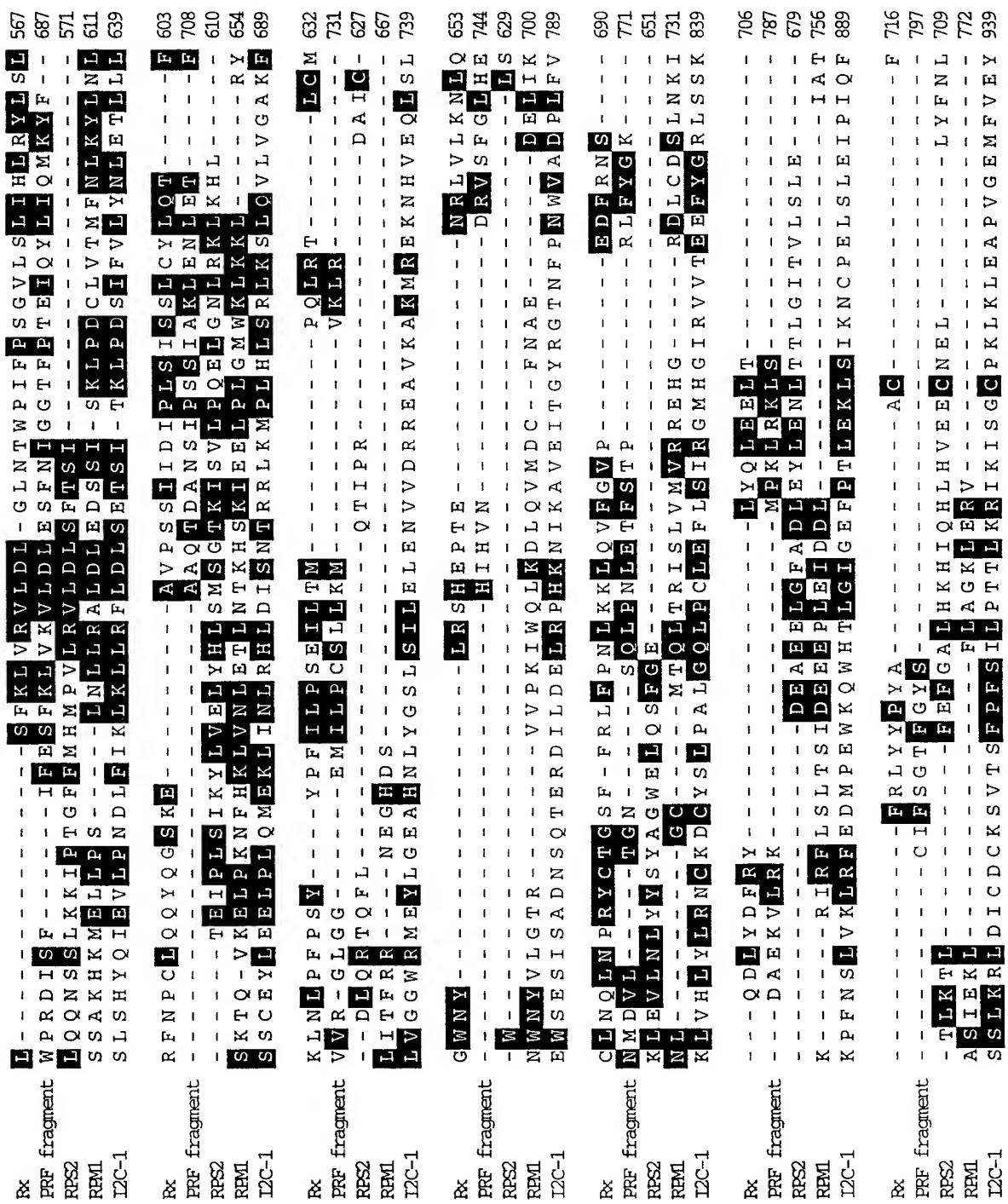

Figure 2...cont

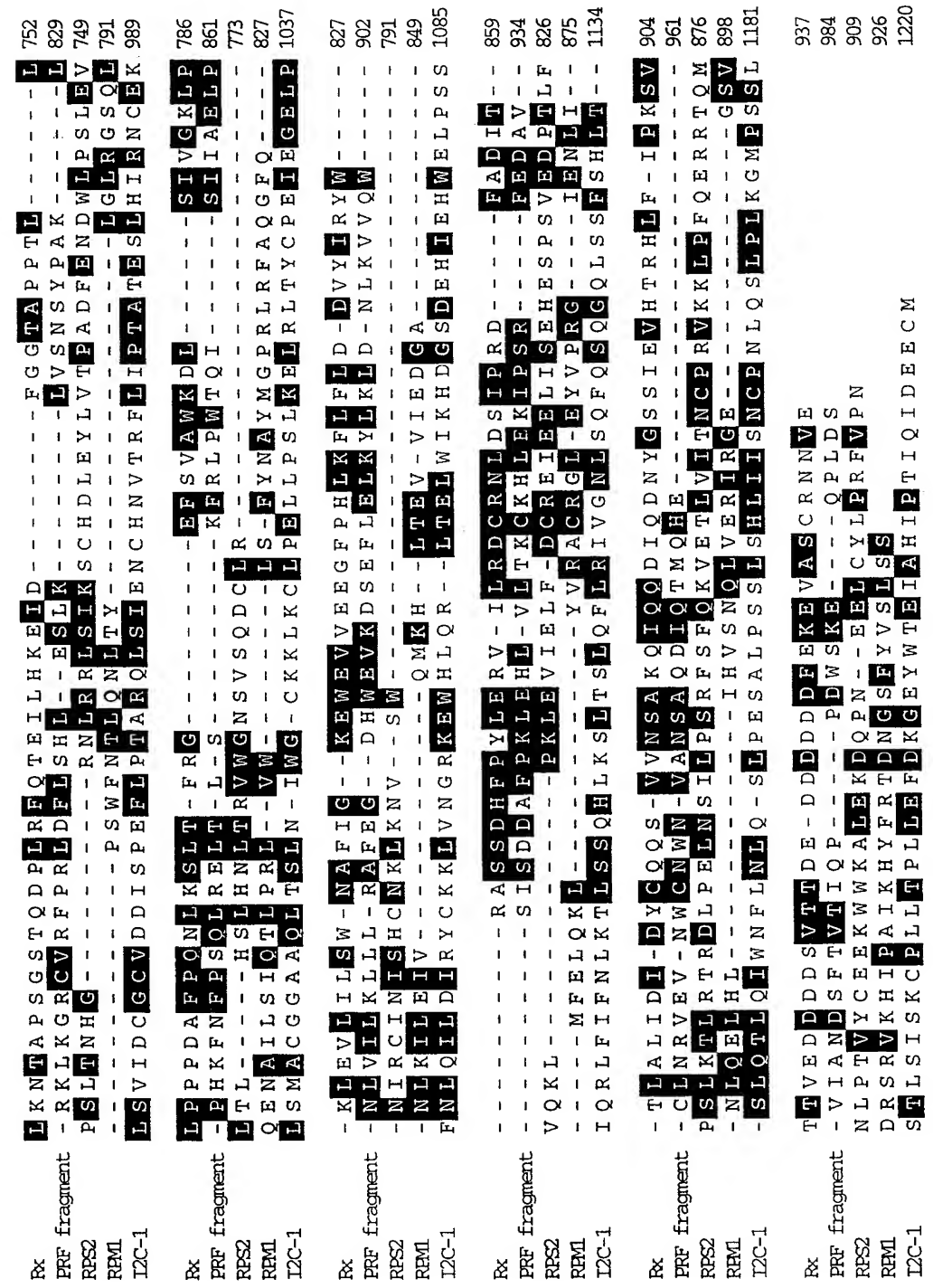

Figure 2 ...cont

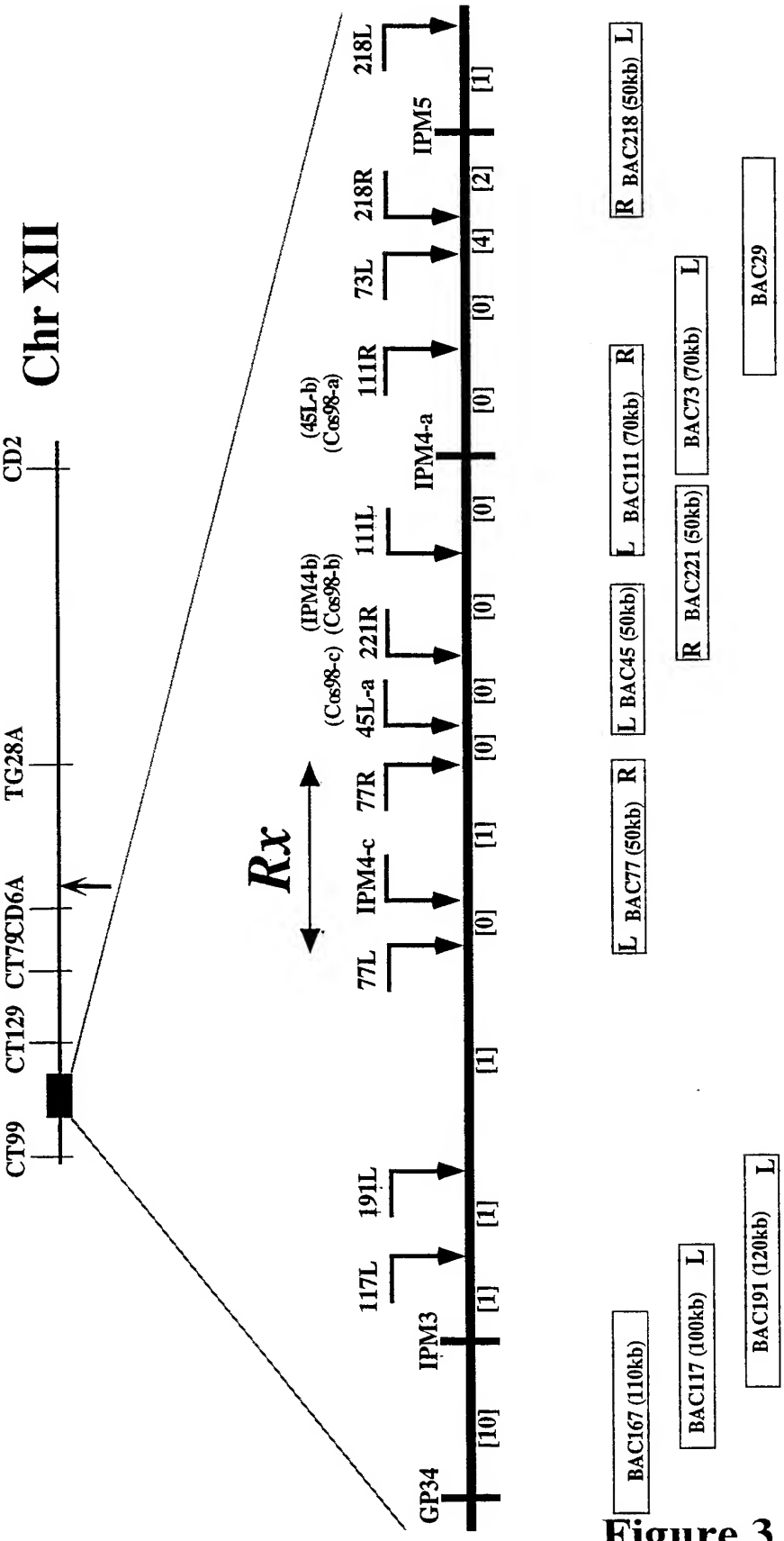
Chr XII
CD2
TG28A
CT79CD6A
CT129
CT99
Rx
GP34
IPM3
IPM4-c
IPM4-a
IPM5
(Cos98-c)
(IPM4b)
(Cos98-b)
(45L-b)
(Cos98-a)
117L
191L
77L
77R
45L-a
221R
111L
111R
73L
218R
218L
[10]
[1]
[0]
[2]
[4]
L BAC77 (50kb) R
L BAC45 (50kb)
L BAC111 (70kb) R
R BAC218 (50kb) L
R BAC221 (50kb)
BAC73 (70kb) L
BAC29
BAC167 (110kb)
BAC117 (100kb) L
BAC191 (120kb) L

Figure 3

(a)

IPM4 FPQNLKSLTFRGEFFLAWKDLSIVGKLPKLEVLKLSYNPFKGEEWEVVAEGFPHLKFLFLD
PRF FPSQLRELTL-SKFRLPWTQISIIAELPNLVILKLLLLRAFEGDHWEVKDSEFLELKYLKLD

IPM4 KVYIRYWRASSDHFPYLERLFLSDCYFLDSIRPDFADITTLALIDITRCQQSVGNSAKQIQ
PRF NLKVVQWSISDDAFPKLEHLVLTKCKHLEKIPSRFEDAVCLNRVEVNWCNWNVANSAQDIQ

(b)

73L LPKFLPTFVNFT-SLRVLDLSVNYFNATIPSWLFNTSHNLVYLNLSRSQLNGSLPNAFGNM
Cf-2.1 LASSVPEEIGYLRSLNVLDLSENALNGSIPA-SFGNLNNLSRLNLVNNQLSGSIPEEIGYL
Cf-9 NFDGGLEFLSFNTQLERLDLSSNSLTGPIPSNISGLQ-NLECLYLSSNHLNGSIPSWIFSL

73L SSLRVLDLSGNSIRGNLSHSFEKMSSVSFLNLSRNSFTGYLP
Cf-2.1 RSLNVLDLSENALNGSIPASFGNLNNLSRLNLVNNQLSGSIP
Cf-9 PSLVELDLSNNTFSGKIQE-F-KSKTLSAVTLKQNKLKGRIP

Figure 4

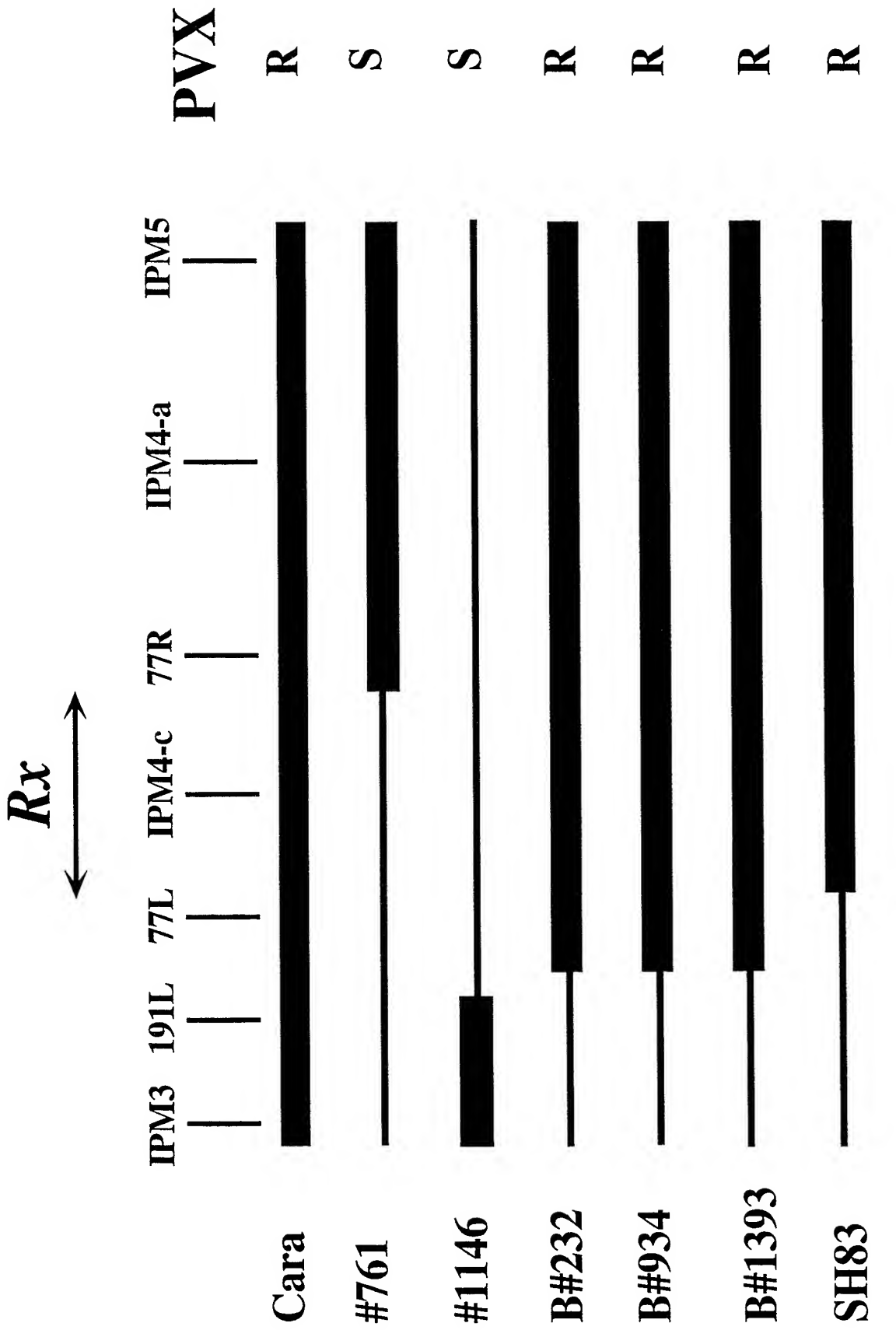
Rx
IPM3
191L
77L
IPM4-c
77R
IPM4-a
IPM5
PVX
Cara
R
#761
S
#1146
S
B#232
R
B#934
R
B#1393
R
SH83
R

Figure 5

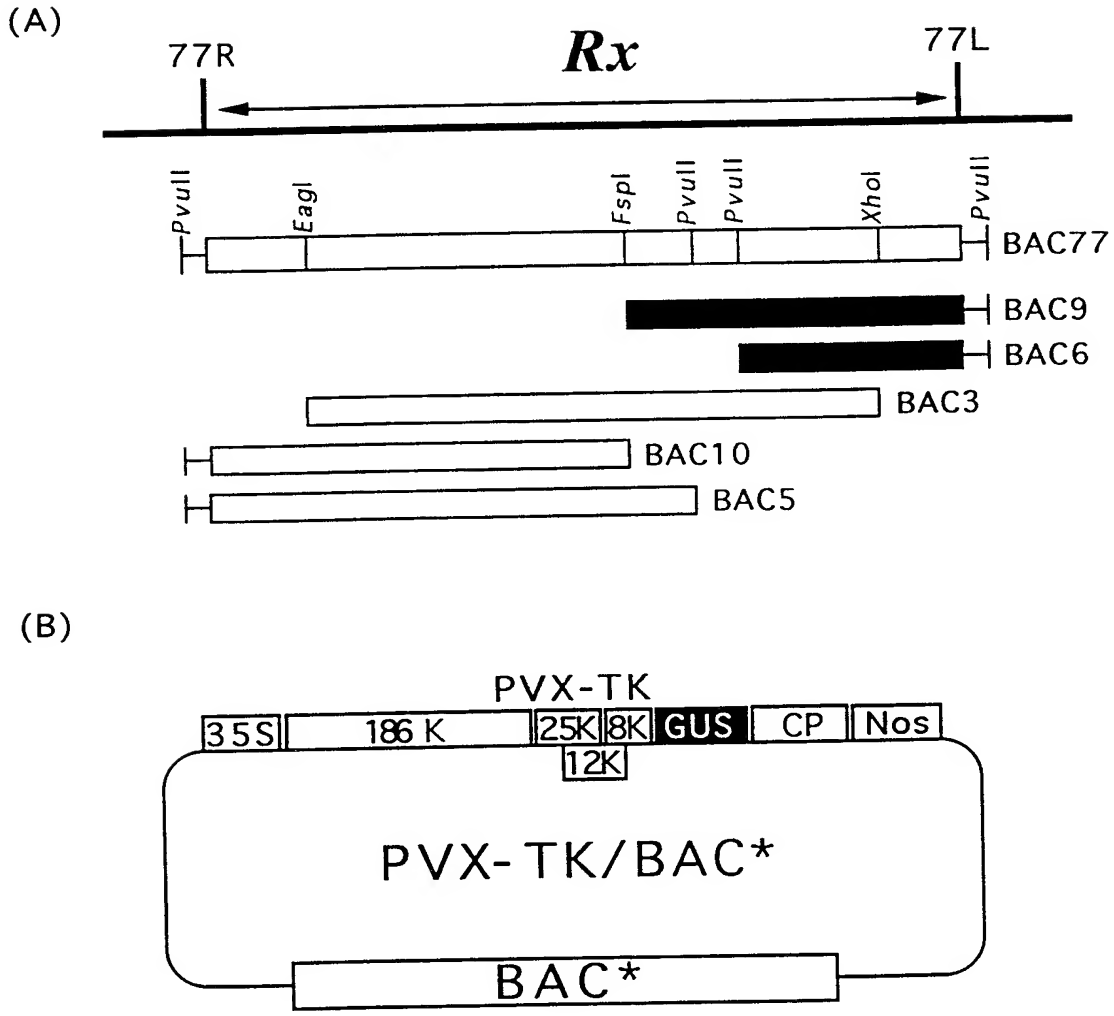
(A)
77R
Rx
77L
PvuII
EagI
FspI
PvuII
PvuII
XhoI
PvuII
BAC77
BAC9
BAC6
BAC3
BAC10
BAC5
(B)
PVX-TK
35S
186 K
25K
8K
GUS
CP
Nos
12K
PVX-TK/BAC*
BAC*

Figure 6

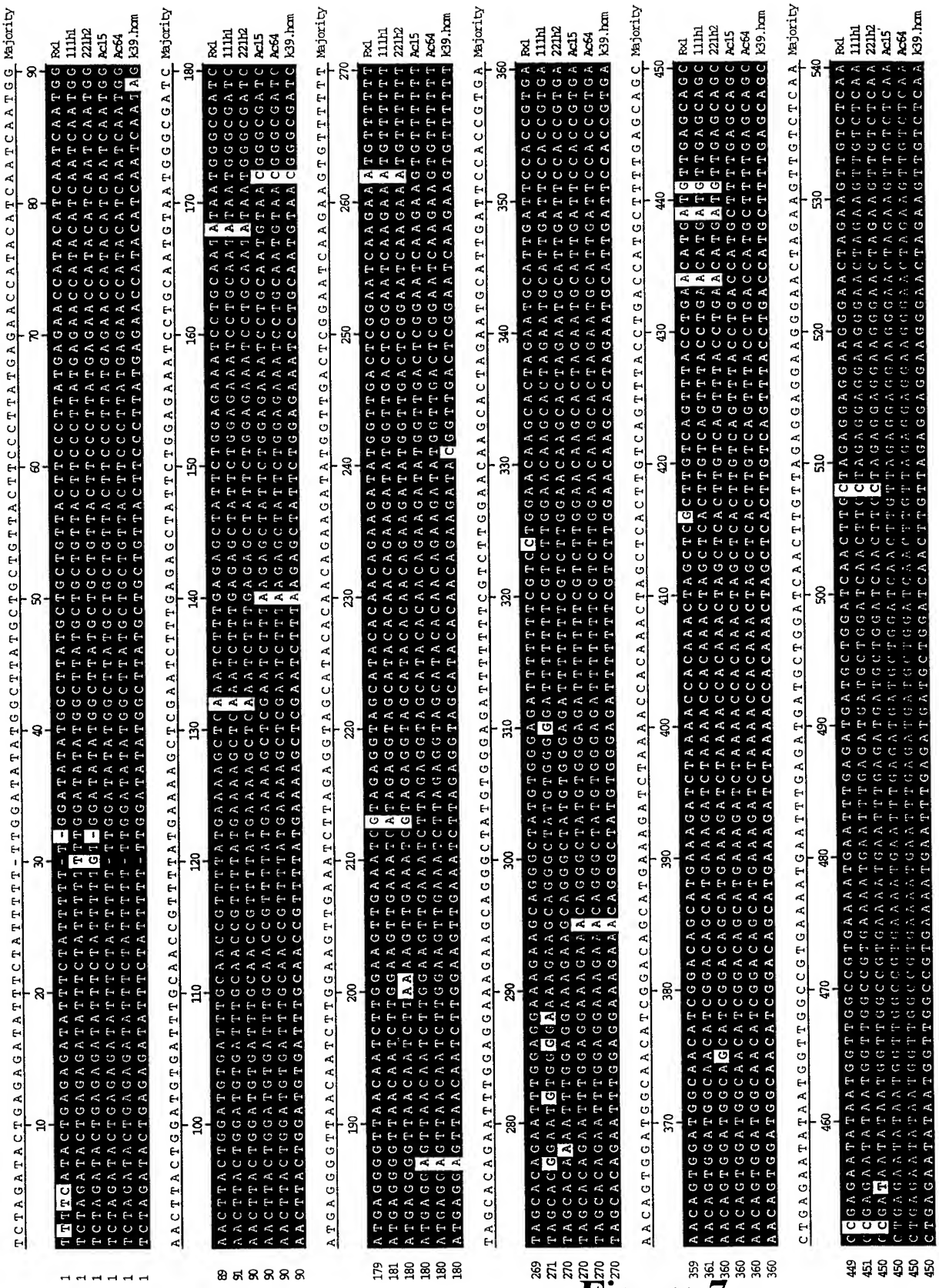

Figure 7 a

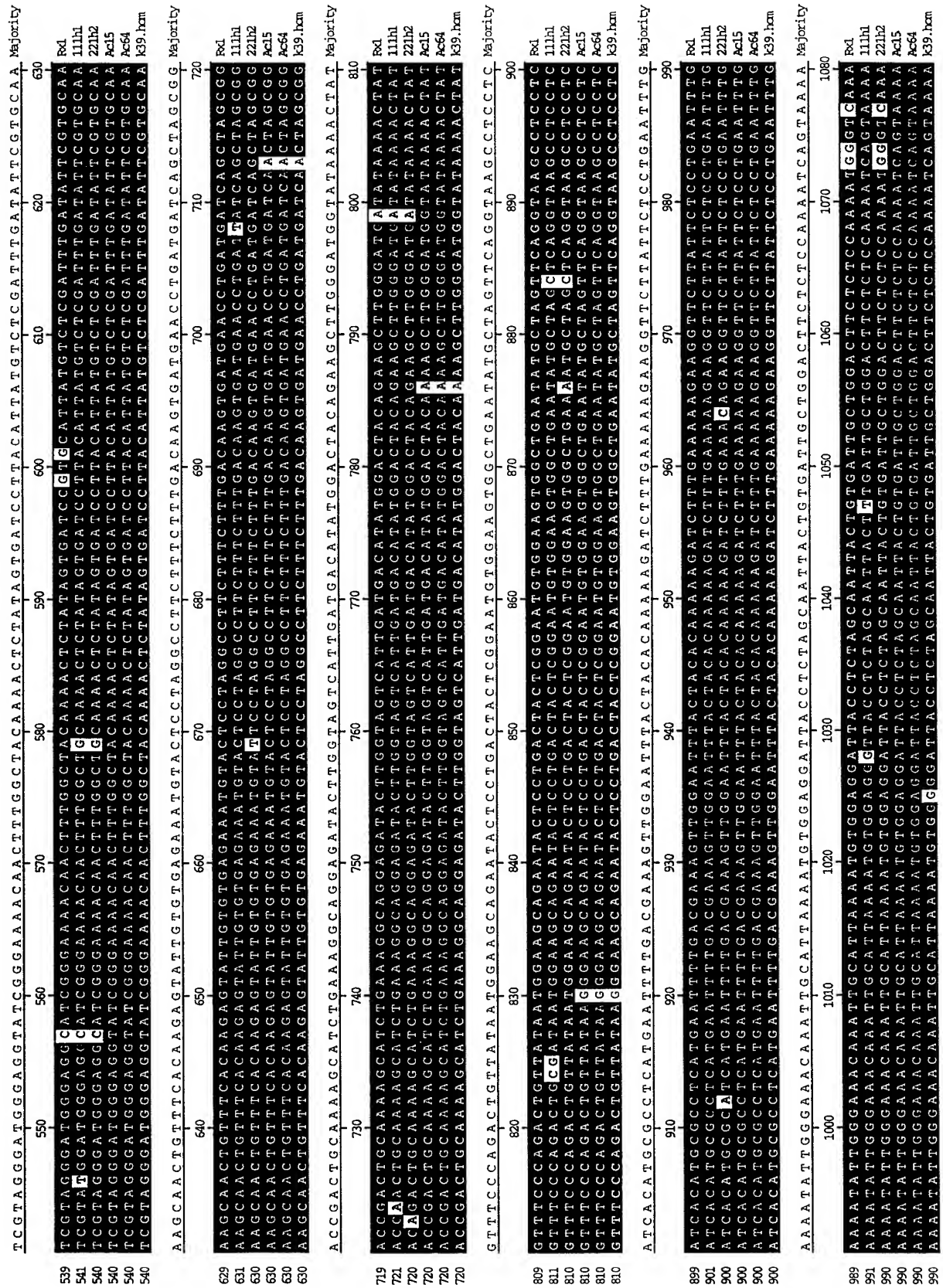

Figure 7a ... cont

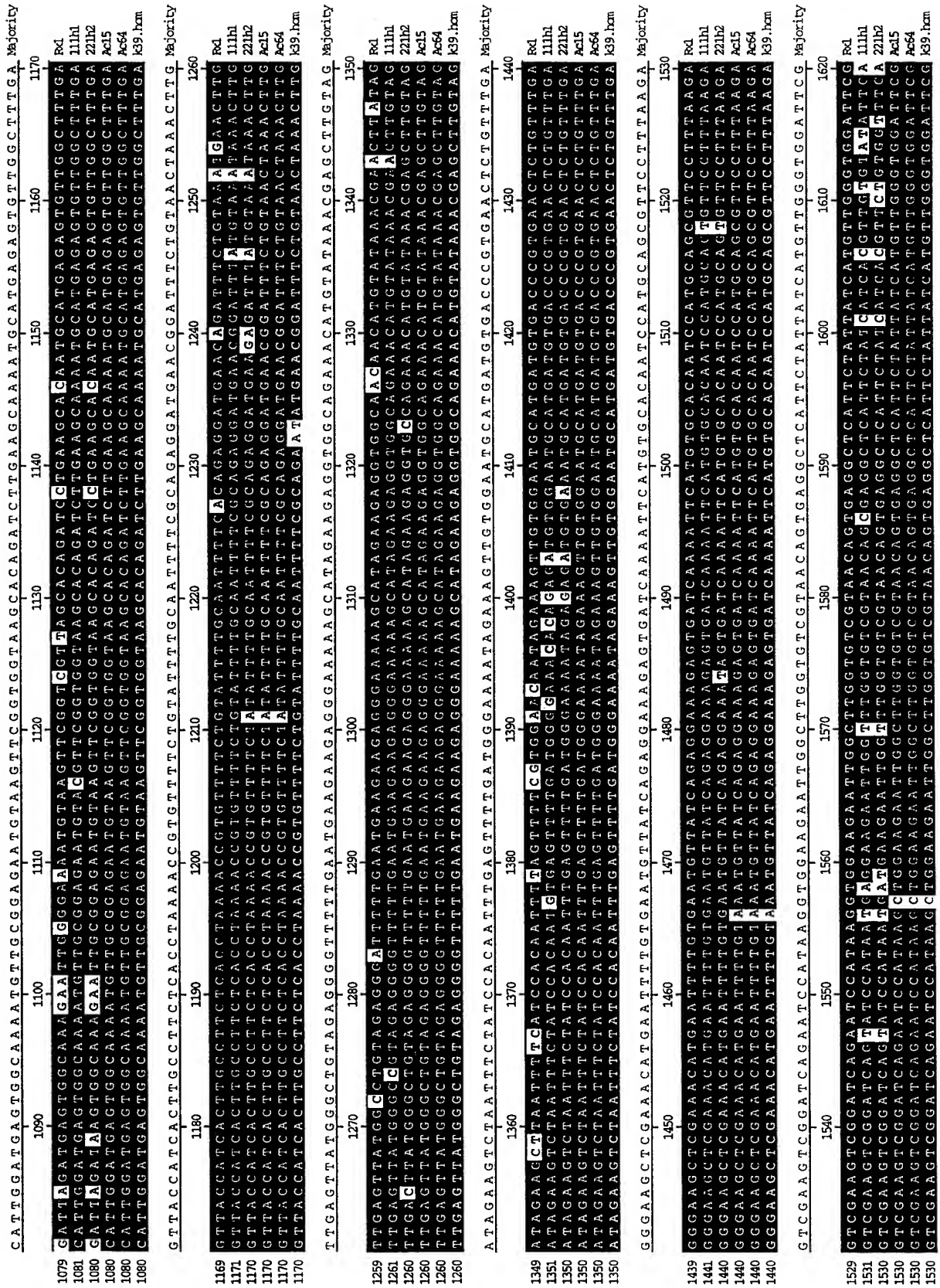

Figure 7a ... cont

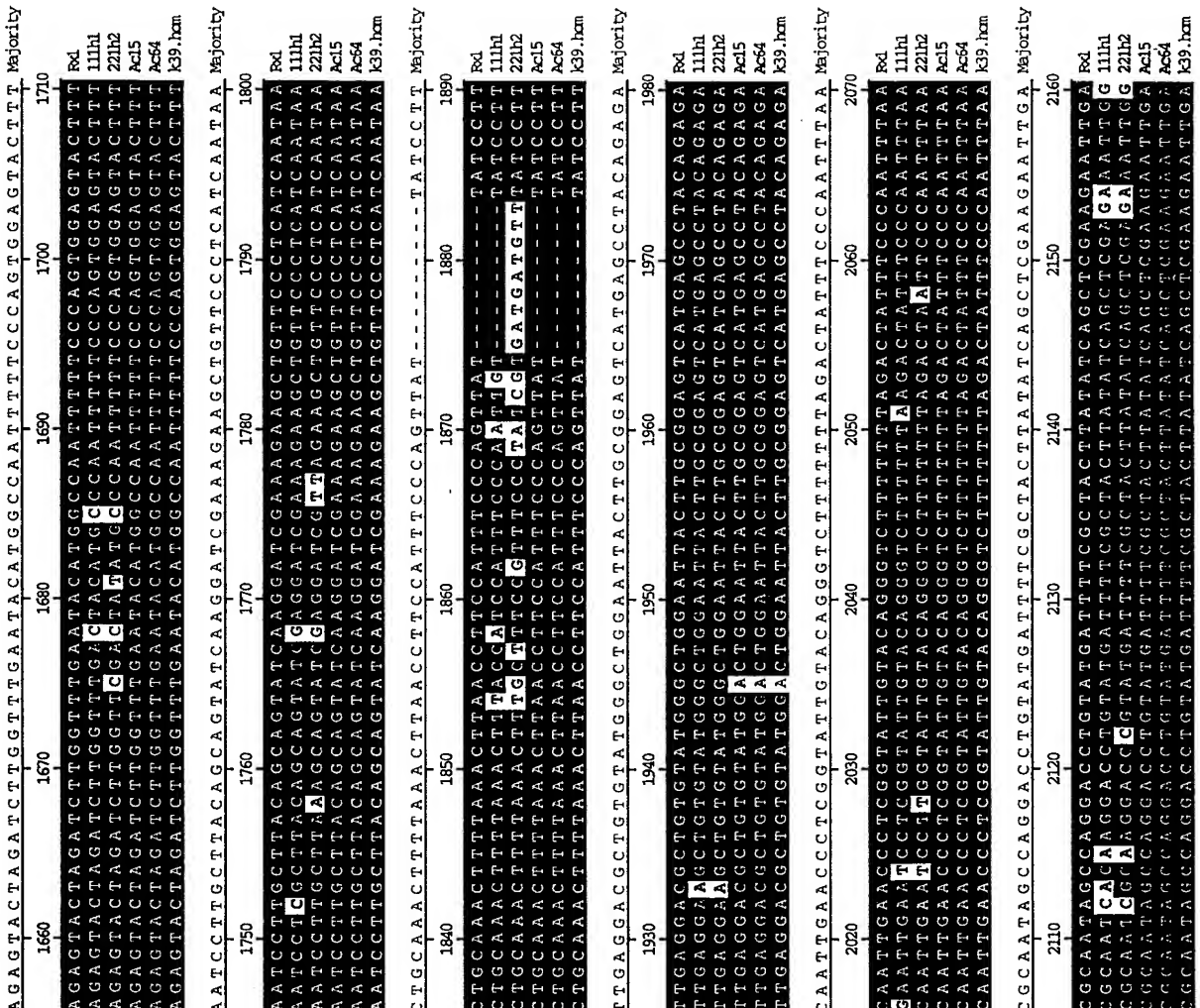

Figure 7a ... cont

Figure 7a ... cont

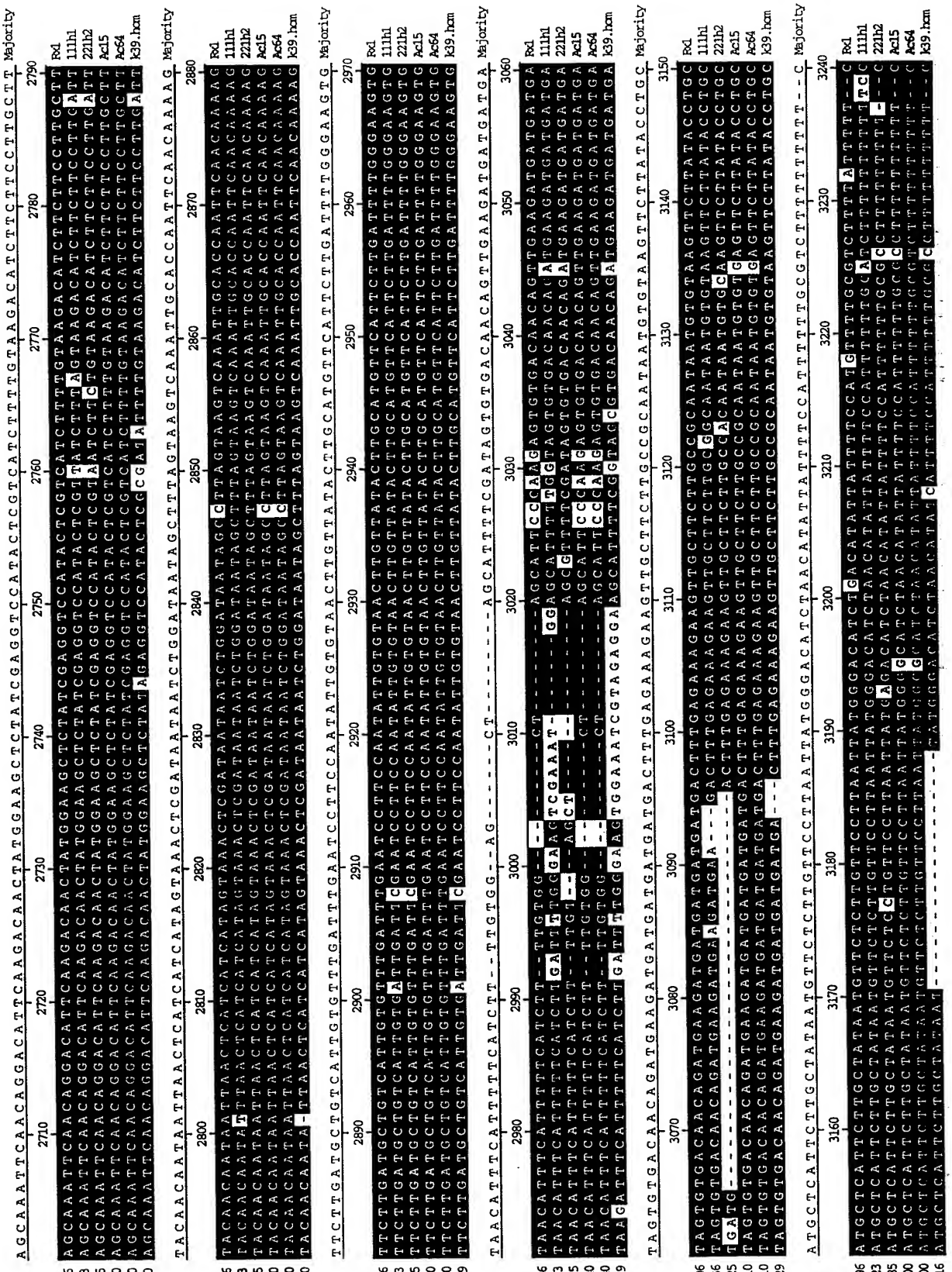

Figure 7a ... cont

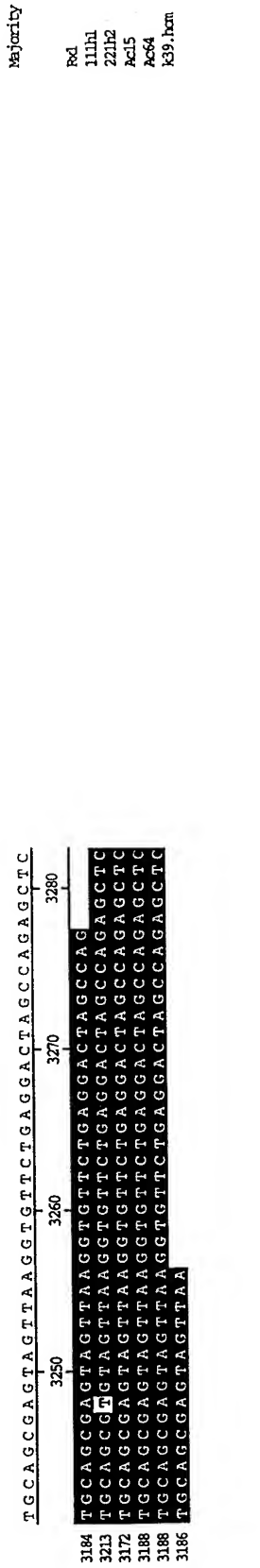

Figure 7a ... cont

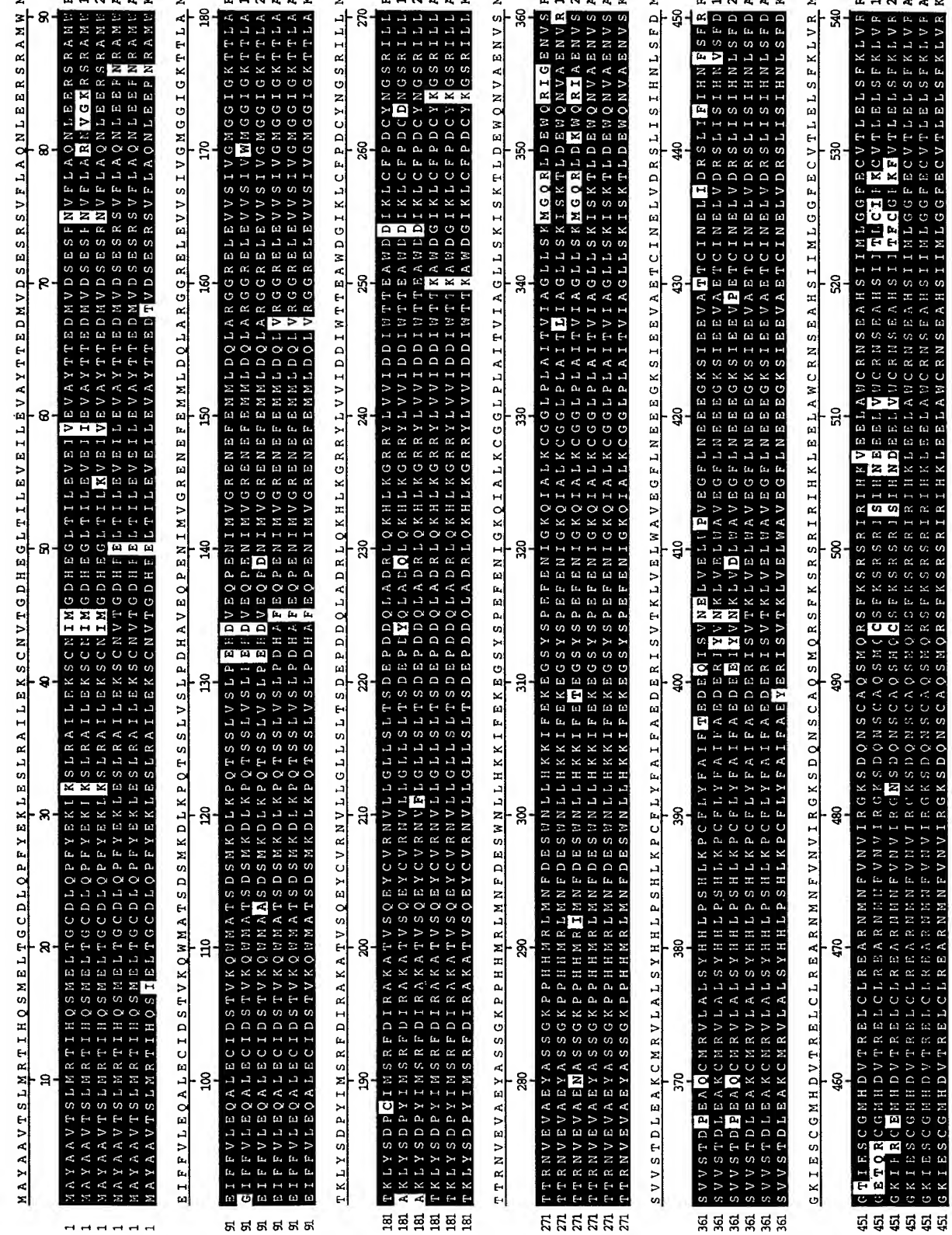

Figure 7b

Figure 7b ... cont

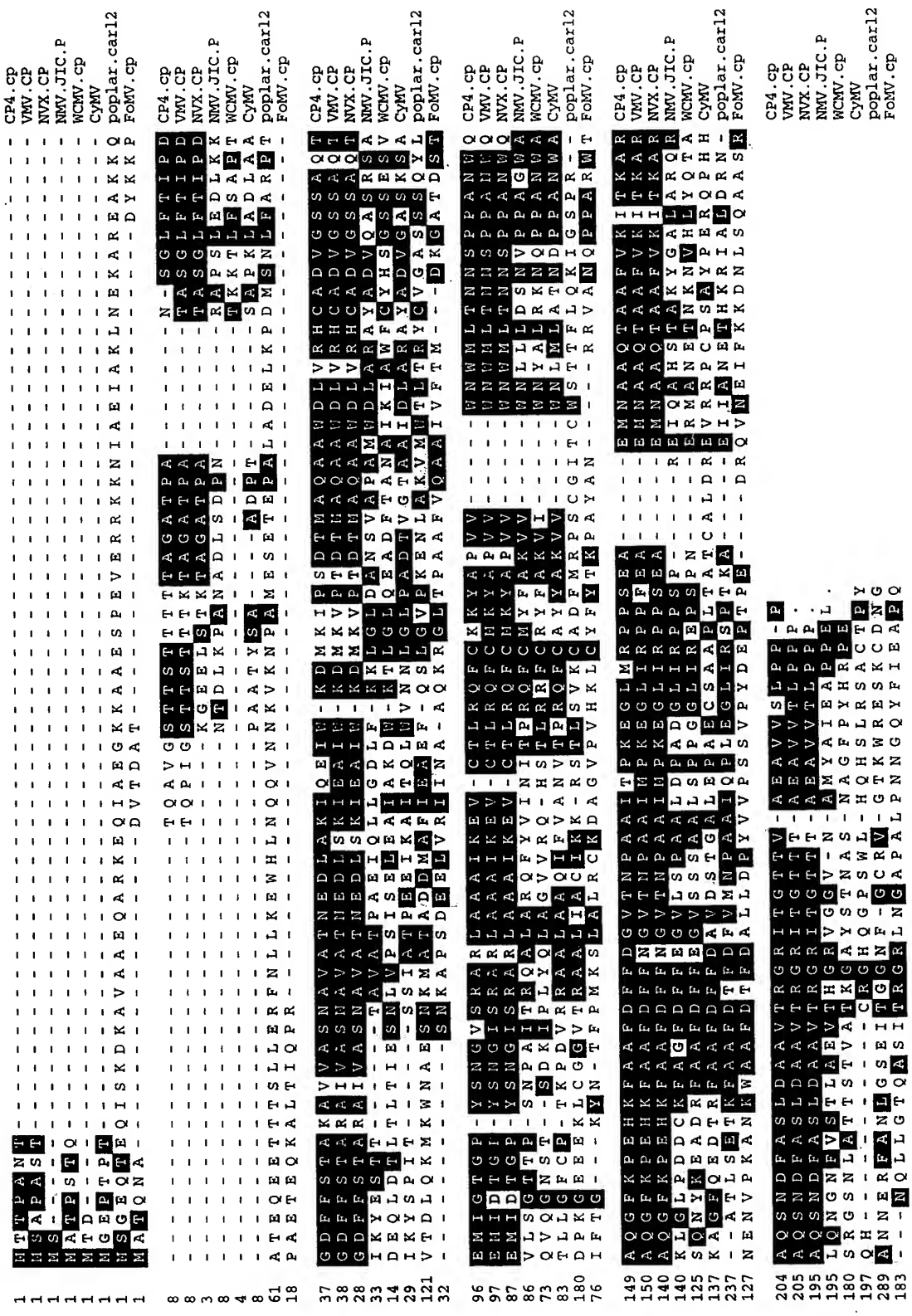

Figure 8